Biologically active constituents of chrysanthemum parthenium.

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BIOLOGICALLY ACTIVE CONSTITUENTS

of

CHRYSANTHEMUM PARTHENIUM

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July 1982

ABSTRACT

The use of the leaves of *Chrysanthemum parthenium* in the prophylaxis of migraine has received much recent publicity. In view of widespread consumption of the plant with apparently efffective results it was considered desirable to investigate this claim.

The present work thus involved successive extraction of freeze-dried leaves with light petroleum, chloroform, methanol and water. Each of these extracts was separately tested for spasmoltyic activity using an *in vitro* preparation of guinea pig ileum. Agonists used in the test were acetylcholine, 5-hydroxytryptamine and histamine. The light petroleum and chloroform extracts showed 100% inhibition of all three agonists at a concentration of 10^{-4} g/ml whereas the methanol and water extracts were devoid of activity. Successive chromatographic separation of the active extracts allowed isolation and purification of some active constituents. These all proved to be sesquiterpene lactones, a class of secondary metabolite which is widespread in the Compositae.

Some of these active materials were already known in the plant but others, including the most active, are apparently new compounds. The structures of these materials have been elucidated by chemical and spectroscopic means, particularly hydrogen-1 and carbon-13 nuclear magnetic resonance spectroscopy. All the active substances may be considered as derivatives of parthenolide, the major germacranolide of the plant, but one (the most active) has a novel trimeric structure.

INDEX

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I am greatly indebted to the following people:-

Dr P J Hylands, Chelsea College, London, for his supervision, advice and encouragement

Dr E S Johnson, Kings College, London, for help with the pharmacological work, valuable discussion and the clinical work

The technical staff of the Pharmacognosy Department, Chelsea College, London, for all their help

Dr G Hawkes, Queen Mary College, London, for 400 MHz ^{1}H NMR and 100 MHz ^{13}C NMR spectra

Mr G McDonough, Chelsea College, London, for 200 MHz ^{1}H NMR spectra

The staff of the mass spectrometry unit, Chelsea College, London, and Mr D Carter of the London School of Pharmacy, London, for mass spectra

Chelsea College for my research grant

The Curator, The Chelsea Physic Garden for the plant material

My parents and Peter for their constant encouragement.

Chrysanthemum parthenium

FOREWORD

In 1978 and 1979 several articles appeared in the national and provincial press about the efficacy of Chrysanthemum parthenium in migraine.[1-4] This was not a new discovery.

Chrysanthemum parthenium Bernh. belongs to the tribe Anthemideae of the family Compositae. It is found throughout Europe both wild and cultivated in and near gardens, walls and rivers. It is a perennial plant growing to a height of 14 to 45 cm with strong-smelling, greenish-yellow, bipinnate leaves.[5] The flower-heads are arranged in a loose terminal corymb the central disc florets being yellow and the outer ray florets white. A double variety is usually cultivated in gardens for ornamental purposes.[6a]

Feverfew, to give the plant its common English name, is perhaps a corruption of featherfew (relating to the form of the leaves) or more attractively as far as the present study is concerned, of the word febrifuge meaning that which dispels fever.[5,6a] One could expect therefore to find folk lore use of the plant in cases of fever. Richard Banckes, writing in his herbal of 1525 considered it indispensable. He said it was,

> 'Good to assuage the access (ague or fever), quotidian (fever recurring daily) or cramp'.[7]

John Gerarde, in 1597, said of the plant,

> 'It is used both in drinks, and bound to the wrists with bay salt and the powder of glasse stamped together, as a most singular experiment against the ague'.[8]

It is similarly referred to fifty years later by John Parkinson[9] and later by Nicholas Culpeper.[10]

A very similar plant, however, Anthemis nobilis L., or Roman

chamomile, is mentioned more frequently as a febrifuge and, particularly as far as this study is concerned, as an antispasmodic as well as in migraine.[6b,11] Unlike feverfew, there is no record of current use of this plant in migraine and so it could be that these plants have been confused in the past. In fact, the 1934 British Pharmaceutical Codex[12] states that feverfew could be and often was substituted for the Roman chamomile. However, one can find references in the literature which allude to the usefulness of feverfew in headache and migraine.[6a,8-10,13]

John Gerarde says,

> 'Feverfew dried and made into powder, and two drams of it taken with honie or sweet wine purgeth by siege melancholic and flegme; wherefore it is very good for them that are giddie in the head, or which have the turning called vertigo, that is a swimming and turning in the head'.[8]

Parkinson[9] and Culpeper[10] only mention C. parthenium being used externally for headache, for example,

> 'It is very effectual for all paines in the head, coming of a cold, caufe, as Camerarius faith, the hearbe being bruised and applied to the crowne of the head'.[9]

More recently feverfew is mentioned as being useful in hysterical complaints and in allaying sensitiveness to pain in highly nervous subjects.[6a,13] This may be of some relevance since migraine sufferers are frequently of a nervous disposition.

The plant is quoted repeatedly as having some action on the female reproductive system,[6-15] most frequently being said to expel the placenta and still-born children and to induce abortion. Thus, Gerarde says,

> 'it procureth womens sickness with speed; it bringeth forth the afterbirth, and the dead childe, whether it be drunke in the decoction or boiled in a bath and the woman sit over it'.[8]

It is also reported to cause abortion in cows.[16]

As well as the current use of C. parthenium in migraine it has been found to be beneficial to many sufferers of rheumatoid arthritis.[1,2,17] Gerarde reports,

> 'Dioscorides also teacheth that it is profitable applied to Saint Antonies fire, to all inflammation and hot swellings'.[8]

and Margaret Grieve[6a] that it gives relief to the face-ache or earache of a rheumatic person.

In common with many medicinal plants feverfew was also used as a tonic,[6a,14] this probably being due to the presence of extremely bitter sesquiterpene lactones.

Other uses are, as an expectorant,[8-10] for the prevention of insect bites,[6a,11] for the removal of freckles[9,10] and 'an especiall remedy against opium, that is taken too liberally'.[9,10]

Interest in feverfew was suddenly reawakened in 1978 in Wales.[1-4] Mrs Ann Jenkins of Cardiff, wife of the National Coal Board's Chief Medical Officer, had suffered with severe migraine from her teens. Conventional treatment was not successful in her case but in 1974 an elderly Welsh miner heard of Mrs Jenkin's plight and sent her a clump of the plant with instructions to eat some of the leaves every day. After 6 months she did not have a headache for a whole month. Previously she had had attacks every 10 days. After 14 months her headaches had stopped and to date have not reappeared (8 years). Mrs Jenkins was so impressed she told friends who suffered from migraine about the plant. It is now believed that many thousands of people in this country are taking feverfew for migraine and perhaps the same number, if not more, are taking it for arthritis.[1,2,17] Indeed Mrs Jenkins, who is mainly responsible for the recent widespread use of

the plant found her rheumatic pains, which had previously made it agonising for her to drive a car, had also disappeared. The case history of Mrs Jenkins looks to be typical of those patients deriving benefit from the plant. The effects take several months to appear. Gradually the frequency and severity of the headaches diminish and after some time may disappear altogether.

In view of the possible large scale consumption of feverfew in this country a systematic investigation of the plant was thought to be worthwhile on two counts. Firstly, it seems from patient reports that feverfew is effective and therefore identification and isolation of the active constituents of the plant is desirable. Secondly, it was important to try to establish any possible toxic side-effects for which the plant may be responsible. It has already been reported that mouth ulcers occur[3,17,18] but long term kidney and liver function and blood tests had not been performed on patients taking the plant.

PART I

I N T R O D U C T I O N

1 THE FAMILY COMPOSITAE

The family Compositae contains nearly 1000 genera and about 15000 species. They are divided into two sub-families, Tubuliflorae and Liguliflorae, and 13 tribes, as shown below:[19]

A Tubuliflorae
- 1 Vernonieae
- 2 Eupatorieae
- 3 Astereae
- 4 Inuleae
- 5 Heliantheae
- 6 Helenieae
- 7 Anthemideae
- 8 Senecioneae
- 9 Calenduleae
- 10 Arctotideae
- 11 Cynareae
- 12 Mutiseae

B Liguliflorae
- 13 Cichorieae

2 SECONDARY PLANT METABOLITES IN THE FAMILY COMPOSITAE

The family Compositae is chemically extremely diverse. The combined occurrence of sesquiterpene lactones, acetylenic compounds and inulin-type fructans is almost as characteristic of the Compositae as their capitula inflorescences. However, triterpenes and flavonoids seem to be present in every member and seed oils sometimes contain characteristic fatty acids. Large amounts of derivatives of caffeic acid are known to occur as well as cyclitols, iridoid glycosides, alkaloids, diterpenes, cyanogenic glycosides, essential oils, coumarins and several types of phenolic constituents.[20]

A SESQUITERPENE LACTONES

Two classes of secondary metabolites seem to have been selected for special consideration namely the polyacetylenes, about which

much is written,[21] and the sesquiterpene lactones which are of more recent interest.[22a] Increased appearance of this second class is undoubtedly due to developments in instrumentation leading to easier structural elucidation of the compounds. In 1960 barely a dozen naturally occurring sesquiterpenes had been elucidated whereas today more than 600 compounds are known and the pace of their discovery is quickening all the time.[22b]

To date the vast majority of sesquiterpene lactones belong to the Compositae but this may stem from the intensity with which this family and certain genera in particular such as Artemisia, Ambrosia, Helenium and Vernonia have been examined. Nevertheless the incidence of sesquiterpene lactones in the Compositae is unusually high (Table 1) and their appearance in other families may be attributed to parallelism. Moreover, the distribution of sesquiterpene lactones within the Compositae appears to harmonise, at least in part, with divisions laid down by classical plant taxonomy especially when alterations of the sesquiterpene carbon skeleton are considered.[22b,23]

Biogenetic theory assumes that the biosynthesis of sesquiterpenoids involves modification and/or cyclisation of the pyrophosphate esters of trans,trans-farnesol, cis,trans-farnesol or nerolidol.[24] There is not much evidence for this in higher plants but compared with the great variety of sesquiterpenoid structures arising from such cyclisations, the number of skeletal types so far encountered is quite low. There are four main types of hydrocarbon skeletons, resulting from slightly different cyclisation modes and subsequent rearrangements: germacrane, guiane, elemane and eudesmane (Figure 1).

Table 1

Distribution of sesquiterpene lactones in the plant kingdom[22b]

A Lactones formed by oxidation of 'head' methyl groups	
	Taxa
Compositae	450
Umbelliferae	12
Lauraceae	1
Bursereae	1
Magnoliaceae	5
Hepaticae	4
B Other Lactones	
Amaranthaceae	1
Aristolochiaceae	2
Cannellaceae	2
Lauraceae	2

By far the largest number, typical of the Compositae, are γ-lactones, the formation of which involves oxidation of one of the two methyl groups in the isopropyl 'head' of the farnesol-type precursor to a carboxyl group, oxidation of an adjacent methylene group to a secondary alcohol and eventual ring closure (Figure 2).[22-27]

Costunolide, 1, is the most elementary cyclic sesquiterpene lactone since it retains two of the three double bonds of farnesyl pyprophosphate in the trans,trans configuration. It is a germacranolide with a cyclodeca-1,5-diene ring system. The ending -olide is used to denote a compound possessing a lactone

function.

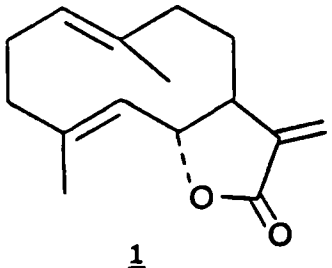

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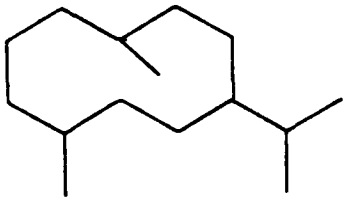
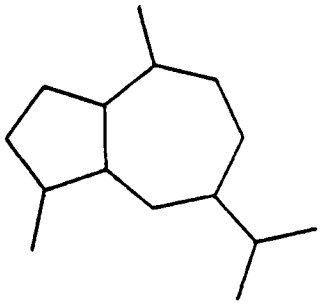
Figure 1

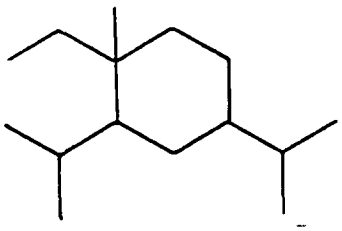
Four main types of sesquiterpene hydrocarbon



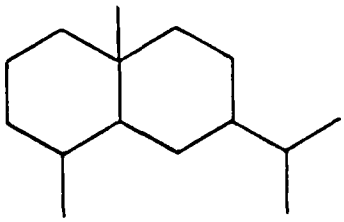
germacrane



guiane



elemane



eudesmane

The details of the process of sesquiterpene lactone formation are not known although two possible biogenetic schemes have been proposed (Figures 3 and 4).[26,28]

The first[26] is a reaction known to occur in plants and may well be responsible for the occasional occurrence of lactones of type a (Figure 3) in those plant groups which contain furanoid sesquiterpenes. Its relevance to the formation of type b lactones so common in the Compositae is not clear however. The second scheme[28a] (Figure 4) therefore is more attractive as a general route to sesquiterpene lactones in the Compositae since

some of the postulated intermediates occasionally accompany the lactone end products. In addition it can be modified to lead to the furanoid sesquiterpenes.

Figure 2

Simplified sesquiterpene lactone biosynthesis

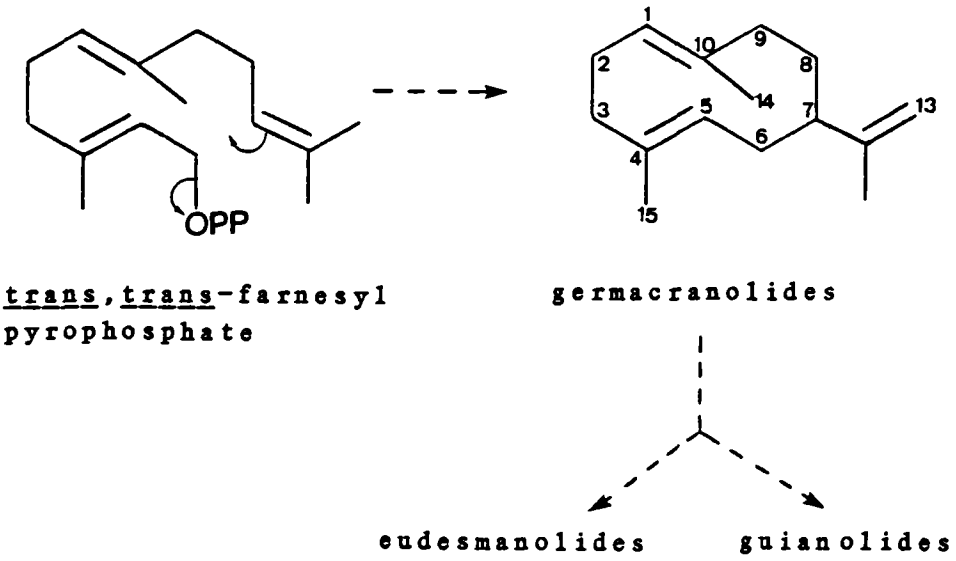
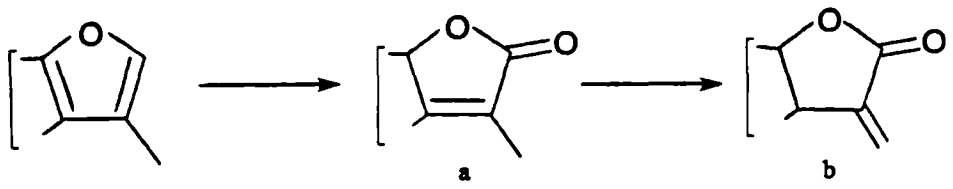


Figure 3

Possible sequiterpene lactone biosynthesis. Proposal 1.

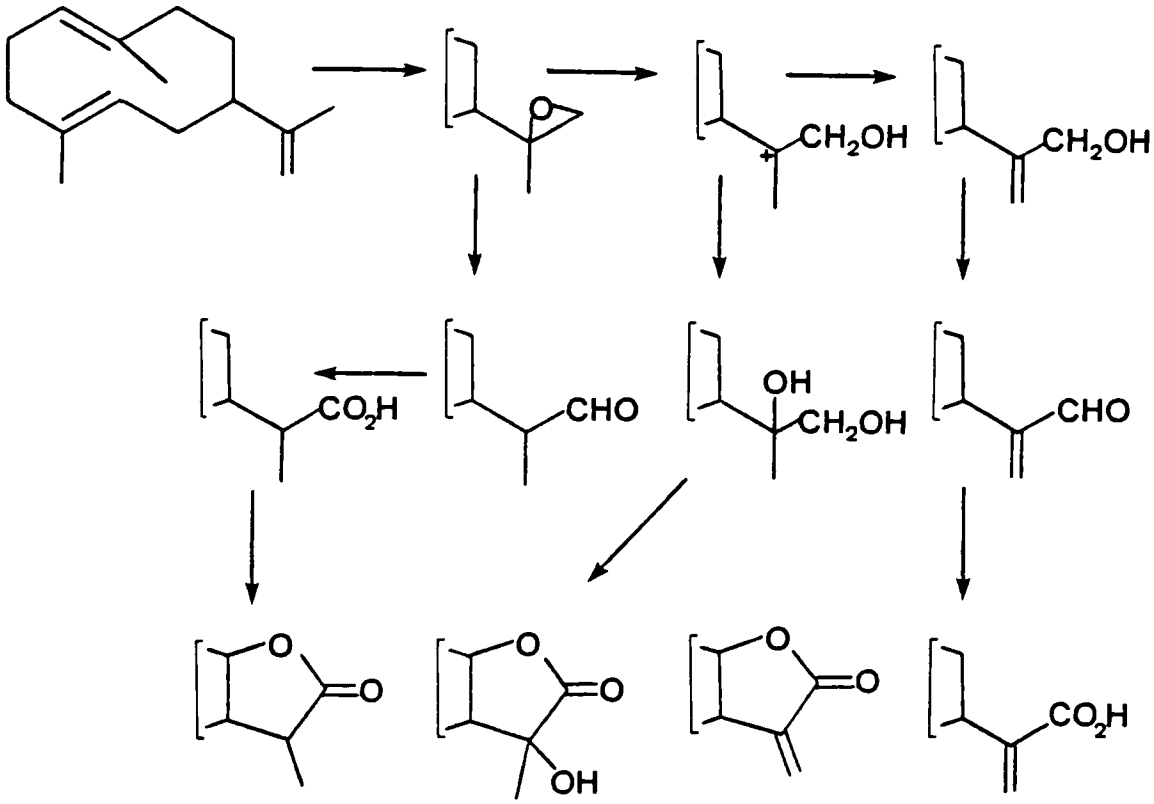


A second, rarer type of γ-lactone results from oxidation of a non-terminal methyl group, for example 2 from Sigubria hodgsonii, the only substance of this kind so far found in the Compositae.[29]

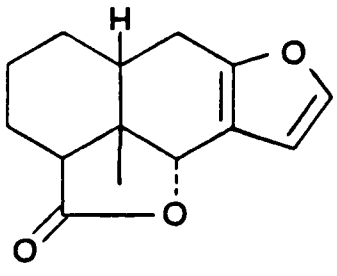
Compounds embodying both types of lactone rings are more frequently found, for example elephantopin 3.[30]

Figure 4

Possible sesquiterpene lactone biosynthesis. Proposal 2.



The germacranolides are probably the biogenetic precursors for



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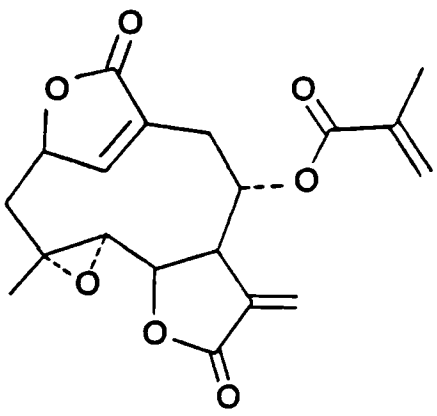

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all the other types of sesquiterpenes (Figure 5).[22c,31a,32] In the figure, for simplicity, initial lactone ring closure at C-6 only is shown although that at C-8 is common.

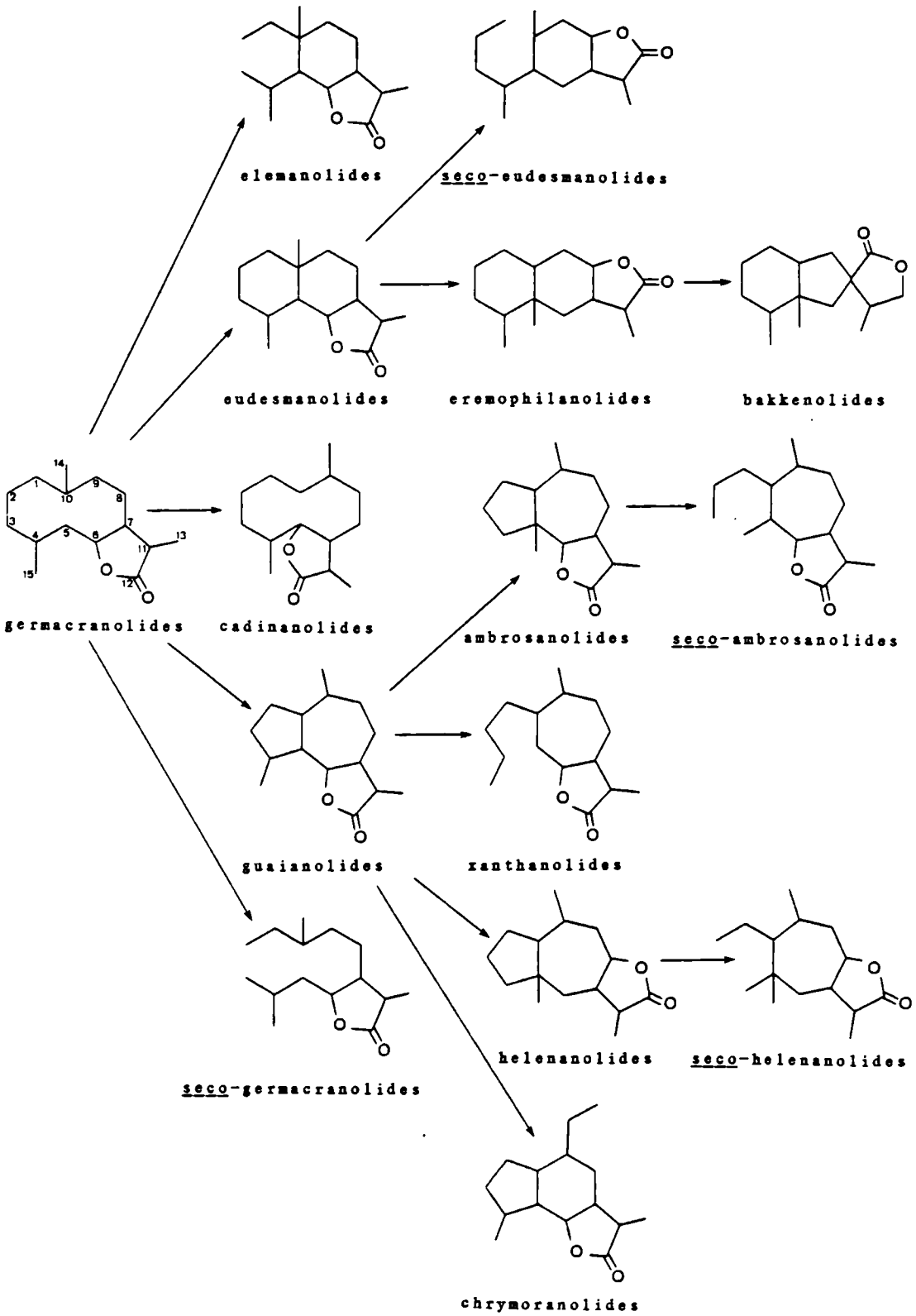
Skeletons in the same vertical columns in Figure 5 are produced, at least superficially, from the precursor farnesyl pyrophosphate by the same number of changes in the carbon skeleton and thus may be said to exhibit the same degree of 'biogenetic complexity'.[22d]

Individual members of a particular class however may differ widely in oxidation state at various sites within the molecule, for example hydroxyl or esterified hydroxyl groups at C-1, C-2, C-3, C-5, C-6, C-8 and C-9, either or both methyl groups oxidised to hydroxymethyl, aldehyde, carboxyl or methylene functions and either or both ring double bonds from farnesyl pyrophosphate transformed into epoxide groups.[28b]

The eudesmanolides, cadinanolides and guianolides appear to be derived by different methods of cyclisation of germacra-1(10),4-dienes or their epoxide derivatives presumably under the control of different enzyme systems.[28b,33] Methyl migrations in the eudesmanolides give rise to the eremophilanolides and those in the guianolides to the ambrosanolides and helenanolides. Oxidative cleavage of the germacranolides, eudesmanolides, ambrosanolides and helananolides is responsible for the respective seco-derivatives. The formation of xanthanolides involves a different method of ring fission from the guianolides. Enzymatically induced ring contraction of the guianolides gives rise to the chrymoranolides and that of the eudesmanolides to the bakkenolides, but so far these have been found in only one species.

Figure 5

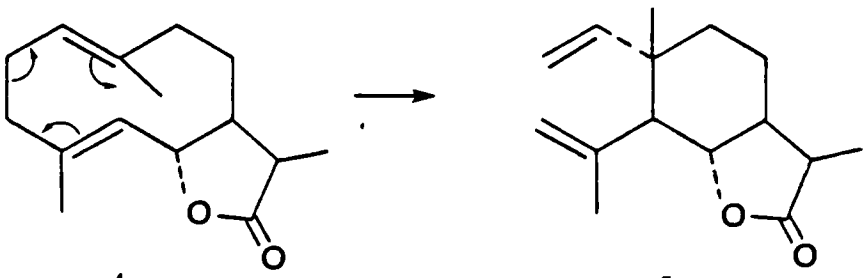
Possible biogenetic relationships of the different skeletal types of sesquiterpene lactones



Many germacranolides undergo Cope rearrangements and so some elemanolides may be artefacts formed during work-up of some plant extracts (see Figure 6).[31b]

Figure 6

Cope rearrangement



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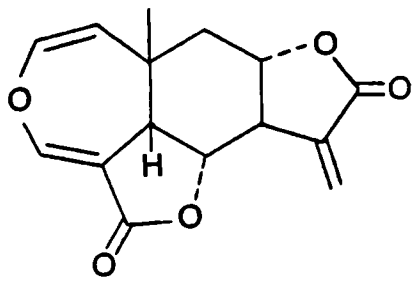
However, the isolation of more complex elemanolides such as miscandenin 6, illustrates the existence of a biological equivalent of the Cope rearrangement in some species.



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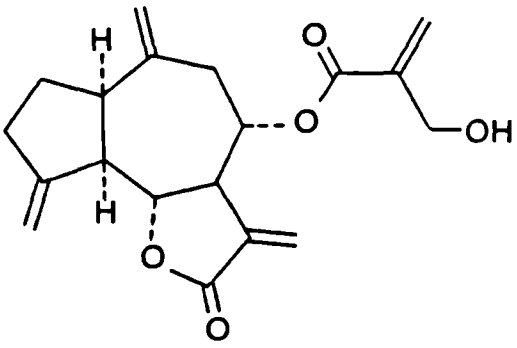
All germacranolides and guianolides may not come from the same biosynthetic pathway because of the discovery of cis,trans-germacra-1(10),4-diene (or heliangolide-type) and trans,cis-germacra-1(10),4-diene (or melampolide-type) compounds.[34,35]

Similarly, probably different biosynthetic pathways account for the large and widely distributed class of cis-fused guianolides, for example cynaropicrin 7[36] and the small group of trans-fused

guianolides such as gaillardin 8.[37]



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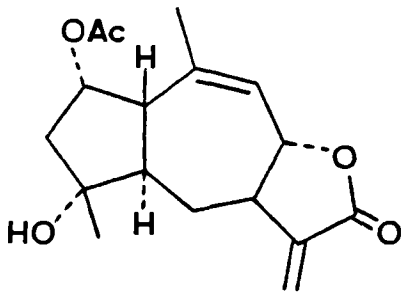

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(a) Anthemideae

Having considered the sesquiterpene lactones in the family Compositae, let us now be more specific and look at the tribe Anthemideae i.e. the tribe in which C. parthenium is placed. Table 2 gives the distribution of sesquiterpene lactones in the Compositae.[22e]

The numbers in the columns represent the taxa from which the lactones of a particular type have been isolated (since some species produce more than one type of lactone, the sum of numbers in a horizontal row generally exceeds the number of taxa).

The Anthemideae, mainly Artemisia and Chrysanthemum, appear to be fairly prolific producers of sesquiterpene lactones. These lactones are mainly germacranolides, eudesmanolides and guianolides, but the tribe also includes two unique examples i.e. arteannin, 9, a cadinanolide from Artemisia annua and chlorchrymorin, 10, a chrymoranolide (a rearranged guianolide) from Chrysanthemum morifolium.[22f]

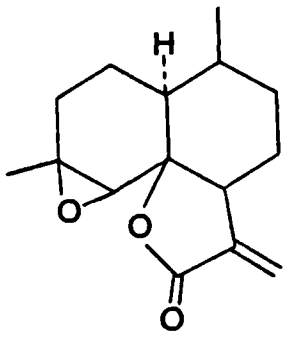
Table 2

Distribution of sesquiterpene lactones in the Compositae[22e]

Tribe	Genera	Taxa	GE	SGE	EL	EU	SEU	ER	BA	GU	XA	AM	SAM	HE	SHE	CA	CH
Vernonieae	4	53	52		3					4							
Eupatorieae	6	30	22	1	2					9		1					
Astereae	1	1								1							
Inuleae	5	16	2		1	8				5	5			2	1		
Heliantheae	29	106	38		3	15	1	2		11	17	35	12	2			
Heleniеae	11	64	8		1	3	1			8				60	8		
Senecioneae	4	8	1					4	2		2			3			
Anthemideae	12	99	33			56				46					1	1	1
Arctoteae-Calenduleae	1	1								1							
Cynareae	11	47	26		3	1				18							
Mutisieae	1	1				1											
Cichorieae	7	10	1			2				8							

Key

GE = germacranolides
SGE = seco-germacranolides
EL = elemanolides
EU = eudesmanolides
SEU = seco-eudesmanolides
ER = eremophilanolides
BA = bakkenolides
GU = guianolides
XA = xanthanolides
AM = ambrosanolides
SAM = seco-ambrosanolides
HE = helenanolides
SHE = seco-helenanolides
CA = cadinanolides
CH = chrymoranolides



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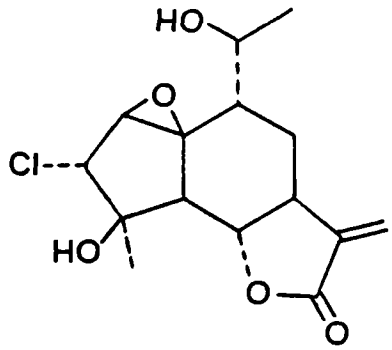

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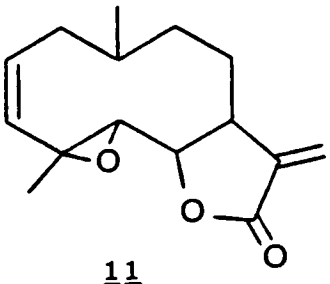
One may reasonably expect therefore that any sesquiterpene lactones present in C. parthenium belong to the germacranolides, eudesmanolides or guianolides. In fact, the only constituents so far reported to be isolated from this plant are parthenolide and santamarine, both germacranolides, chrysartemin A, chrysartemin B, both guianolides, and reynosin, a eudesmanolide (see Part I, 3).

3 CONSTITUENTS FOUND IN CHRYSANTHEMUM PARTHENIUM

A PARTHENOLIDE

In 1959 Sorm et al.[38] isolated a sesquiterpene lactone from C. parthenium which they called parthenolide. Structure 11 was proposed mainly as a result of chemical evidence in conjunction with infrared and ultraviolet spectroscopic studies. The infrared spectrum showed an absorption band characteristic of a γ-lactone at 1767 cm^{-1}. The presence of a band at 1408 cm^{-1}, together with the result of quantitative ozonisation according

to Naves' method[39] (0.42 methylene double bond*) and the tendency of the substance to polymerise readily, lead them to suggest that parthenolide contained an exocyclic double bond in



11

a position α, β- to a lactonic carbonyl group. This structure also explained the high end absorption in the ultraviolet spectrum of parthenolide which was asbent from that of the dihydro-derivative, 12, obtained by hydrogenation of parthenolide using platinum oxide. The infrared spectrum of dihydroparthenolide showed a band at 1774 cm^{-1} due to the lactonic carbonyl but no absorption at 1408 cm^{-1}. The proof for the presence of the $-O-CO-\dot{C}=CH_2$ grouping in parthenolide was completed in 1960[40] by the preparation of a pyrazoline derivative whose infrared spectrum lacked absorption for a methylene double bond.

However, the existence of a double bond in dihydroparthenolide (i.e. of a second double bond in parthenolide) was shown by the uptake of one equivalent of oxygen by dihydroparthenolide to form an oxide, 13, by reaction with perphthalic acid. Total hydrogenation of parthenolide, with an uptake of three molecules of hydrogen, gave hexahydroparthenolide, 14. The infrared spectrum of this gave a band 3599 cm^{-1} for a hydroxyl group in

* This low result is characteristic for substances with a methylene double bond conjugated with a lactonic carbonyl.

addition to that for a lactonic carbonyl at 1750 cm^{-1}. From this it was concluded that a third oxygen was present as an oxide since neither a hydroxyl or ketone is present in the natural compounds.

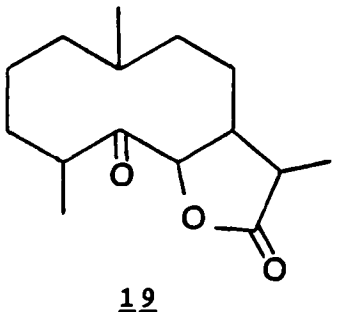
Hexahydroparthenolide, 14, gave rise to chamazulene, 15, on selenium dehydrogenation thus leading Sorm et al. to conclude that parthenolide was a sesquiterpene lactone of the germacrane type.[41]

Parthenolide and its dihydro-derivative, on oxidation with nitric acid, gave a mixture of acids from which β-methyladipic acid, 16, was isolated so proving that at least four carbon atoms of the presumed cyclodecane ring do not carry an oxidised functional group and are substituted with one methyl group.

The character of the isolated double bond was partly explained by the infrared spectra of parthenolide oxide, 13, and dihydroparthenolide oxide, 17. Both these compounds gave bands at 831 cm^{-1} characteristic of a disubstituted cis-1,2-oxide.

Hexahydroparthenolide, 14, on oxidation with chromic acid gave a compound (19) with infrared absorption bands 1785 cm^{-1} for a γ-lactone and 1717 cm^{-1} for a ketone. Since reduction of hexahydroparthenolide with lithium aluminium hydride gave a triol, 18, which on oxidation with periodic acid consumed one mole of reagent, the keto group in 19 must be adjacent to the potential hydroxyl group in the lactone grouping.

This result thus shows that one of the C-O bonds of the oxide ring of parthenolide is attached to carbon 5 of the 4,10-dimethyl-7-isopropylcyclodecane skeleton. As parthenolide gave β-methyladipic acid, 16, on oxidation with nitric acid the oxide

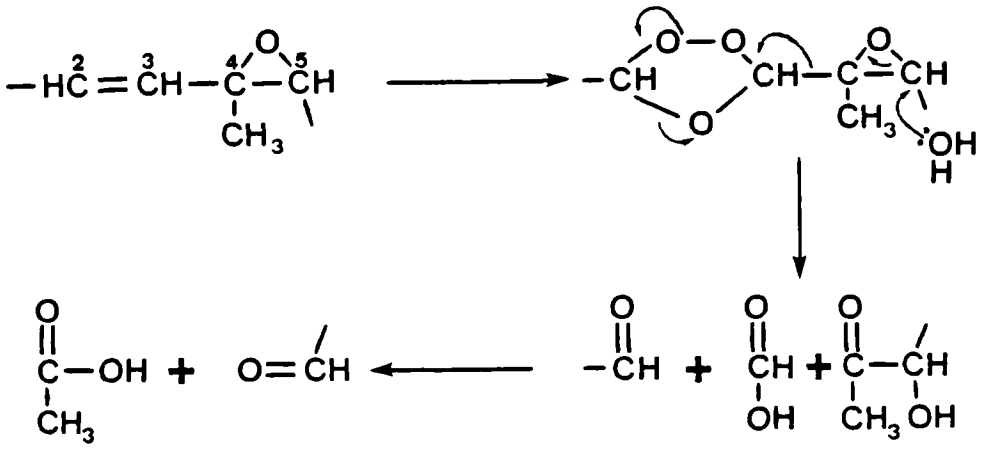


19

ring was presumed to be 3-membered and the double bond so located at position 2. This presumption was apparently verified by isolation of formic acid from the volatile products of ozonisation of dihydroparthenolide and acetic acid on subsequent oxidation of the non-volatile material (Figure 7). The sequence of reactions are summarised in Figure 8.

Figure 7

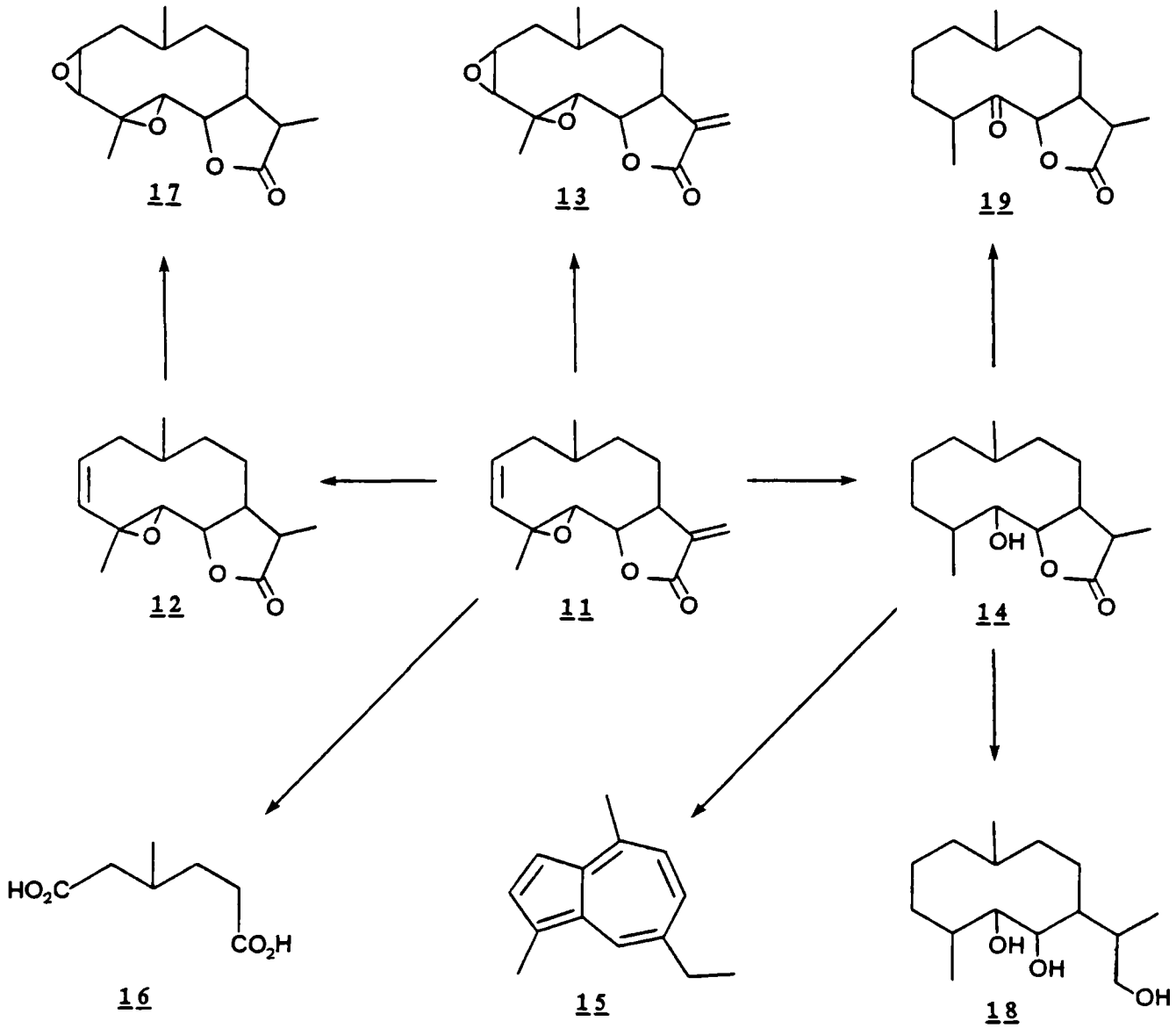
Reactions used in the deductions about the positions of the double bond and epoxide in 11



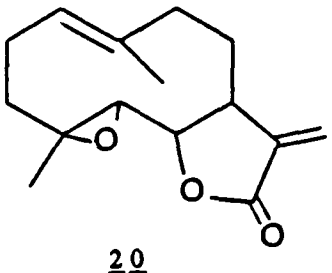
In 1965 however Govindachari et al. isolated parthenolide from

Figure 8

Summary of reactions leading to the structural elucidation of 11



the trunk bark of Michelia champaea[42] (family Magnoliaceae) and with the advent of NMR and new degradation experiments were able to prove the original structure proposed by Sorm to be incorrect. They assigned structure 20 to parthenolide.

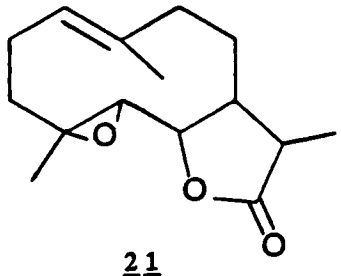


20

The NMR spectrum of parthenolide shows a singlet (3H) at δ1.28 (methyl on carbon carrying oxygen - assigned to C-4 methyl) and a singlet (3H) at 1.72 (methyl on a double bond - C-10 methyl). The double bond therefore cannot be at C-2 as proposed by Sorm.

In addition, reduction of parthenolide using platinum oxide catalyst gave a mixture of stereoisomeric tetrahydroparthenolides. One of these was isolated in a pure state. In the NMR spectrum of this compound the broad signal (1H) at δ5.3 present in the spectra of parthenolide and dihydroparthenolide, 21, had disappeared, and in place of the singlet at δ1.72 (C-10 methyl) a doublet (3H) at δ0.88 (J = 6 Hz) appeared.

Dihydroparthenolide, 21, on reaction with perbenzoic acid gave an epoxy derivative, 22. The signals for the vinyl hydrogens and vinyl methyl were not present but were replaced by a singlet (3H) at δ1.4 due to a methyl on the system $-\overset{|}{\underset{|}{C}}-O-$.



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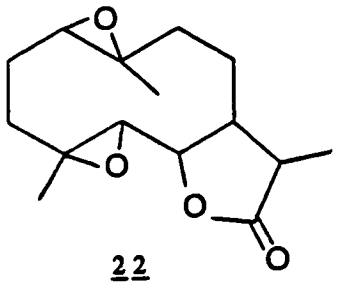
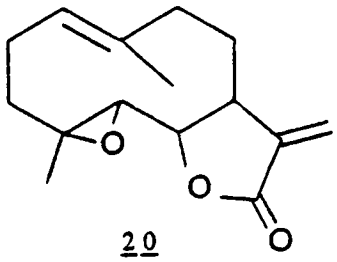


22

Only two structures could be possible for parthenolide consistent with its NMR spectrum: 20 or 23.



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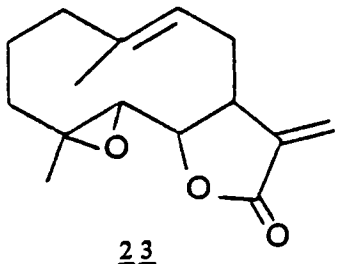
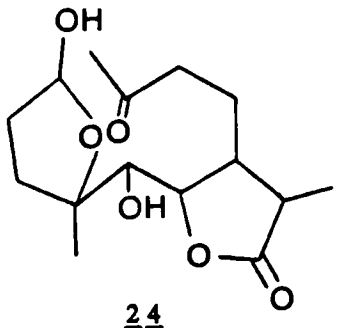


23

That parthenolide has the structure 20 was proved by oxidation of dihydroparthenolide, 21, with sodium metaperiodate to a ketoaldehyde with infrared absorption bands at 2725 (CHO), 1770 (γ-lactone) and 1710 cm^{-1} ($-\overset{O}{\overset{\|}{C}}-R$, CHO). On treatment with dilute hydrochloric acid it yielded a vicinal diol 24 (which gave a positive periodate reaction) by opening the epoxide ring. On the basis of structure 20 for parthenolide the diol could be 24. This material was treated with sodium metaperiodate. The steam-distillate gave levulinic aldehyde, 25, and this could have only resulted if parthenolide has structure 20, with the tri-substituted double bond between C-1 and C-10.



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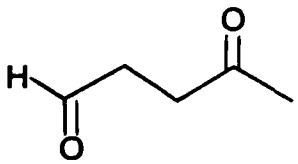
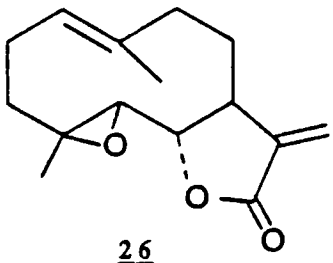


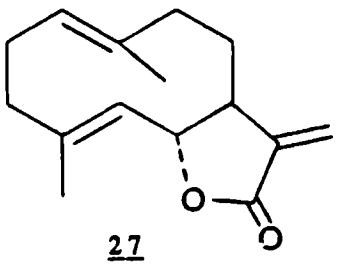
25

The absolute configuration of parthenolide was determined by Bawdekar et al.[43] in 1966 and shown to be as in 26.



26

Parthenolide has a close structural similarity to costunolide, 27, the stereochemistry of which is well established.[44]

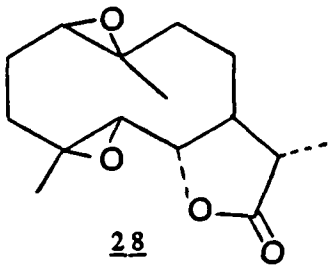


27

Parthenolide can be considered as the 4,5-monoepoxide of costunolide and dihydroparthenolide the 4,5-monoepoxide of dihydrocostunolide. Dihydrocostunolide on treatment with excess perbenzoic acid gave a diepoxide, 28, which was identical with the epoxide of dihydroparthenolide with respect to infrared and nuclear magnetic resonance spectra as well as mixed melting

point. The stereochemistry of parthenolide at C-6 and C-7 and that of dihydroparthenolide at C-6, C-7 and C-11 was therefore established.

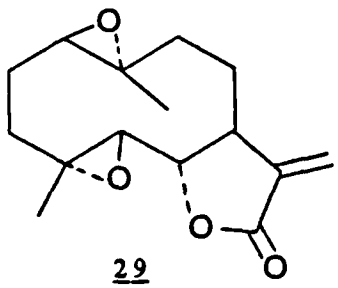
In 1976 Quick and Rogers[45] examined the molecular structure of



28

parthenolide by X-ray crystallography. They found that the two methyl groups are β-orientated, the 1(10)-double bond and the equivalent of the 4-double bond are trans (as expected from biosynthetic considerations) and the ring has a flattened conformation (see Figure 9). The configuration of the asymmetric atoms proved to be 4R, 5R, 6R, 7R.

The geometry of the epoxide showed the molecule to be directly related to costunolide[46] so it follows that the diepoxide described by Bawdekar et al.[43] as derivable from parthenolide must be represented by 29.



29

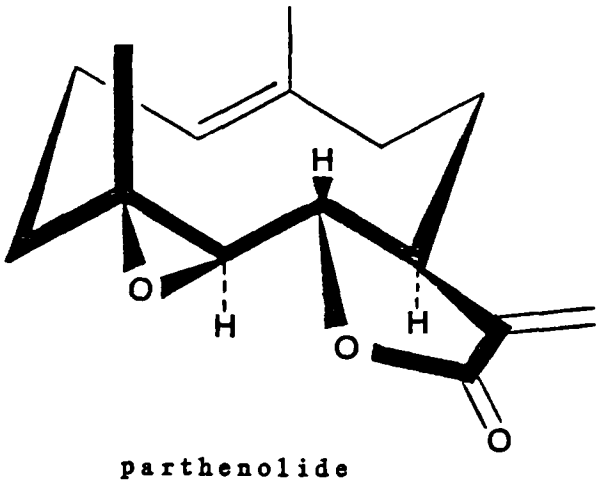
This geometry agreed well with deductions made from nuclear

Overhauser effects observed in the ^{1}H NMR spectra.

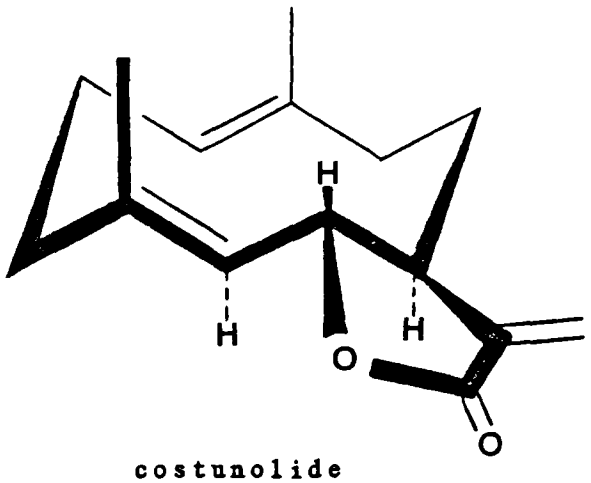
Parthenolide has also been isolated from Ambrosia dumosa.[47]

Figure 9

Conformation of parthenolide and costunolide



parthenolide

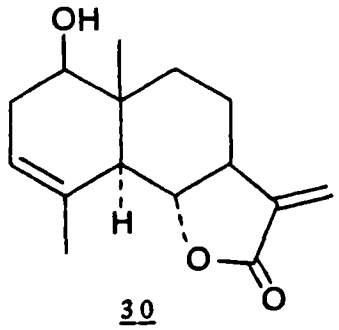


costunolide

B SANTAMARINE

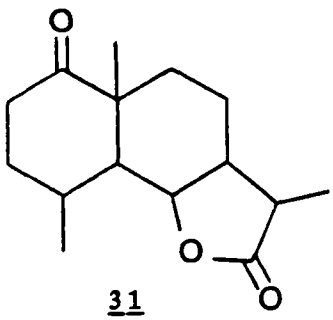
In 1965 Romo de Vivar and Jimeng[48] isolated the eudesmanolide santamarine, 30, from C. parthenium. It was shown to contain a

hydroxyl group by its infrared absorption at 3400 cm^{-1} and the formation of a monoacetate. It was proved to be a secondary



30

alcohol because on oxidation of the dihydro compound with chromic acid a keto derivative, 31, was formed, (IR at 1710 cm^{-1} - six membered ring ketone). This compound gave a positive Zimmermann test indicating that the ketone is flanked by at least one methylene group. There were thus four possible positions for the ketone: C-1, C-2, C-8 and C-9. On mild alkaline treatment, however, a conjugated ketone was not produced so C-8 was eliminated. Similarly, the dihydro derivative, 32, on oxidation gave a non-conjugated ketone, 33, and thus excluded position 2.



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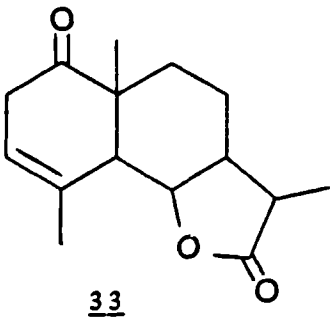
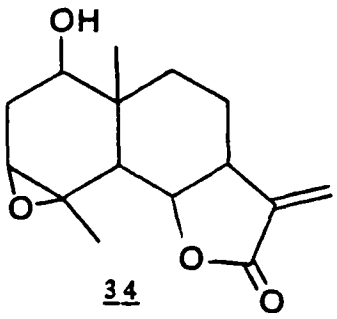


33

Of the remaining two positions C-1 was favoured by the ultraviolet absorption at 205-210 nm characteristic of β,γ-unsaturated ketones. This was confirmed by chromic acid

oxidation of the epoxide, 34, to the keto epoxide, 35, which on alkaline treatment gave an α,β-unsaturated-γ-hydroxyketone, 36 (λ_{max} 215 nm, IR 3600, 1680 cm^{-1}).



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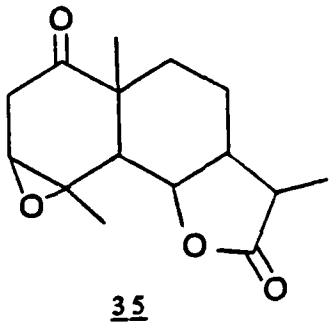

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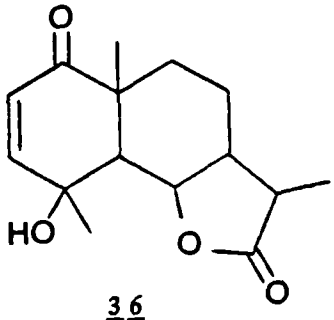


36

On hydrogenation of 31 a good yield of the alcohol, 37, was obtained. Therefore, attack of the C-1 carbonyl by the hydrogen was assumed to occur from the opposite side from C-9 which has a β-orientated methyl group, and the hydroxyl group was therefore given the configuration β-equatorial.

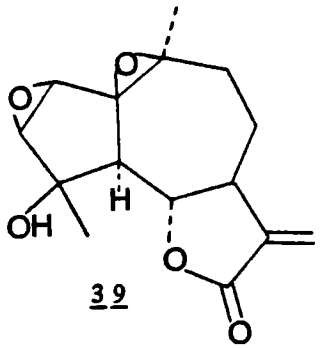
On dehydrogenation santamarine did not produce an azulene so it was assumed to be a eudesmanolide. The skeleton and stereochemistry at C-5, C-6, C-7 and C-10 were established by comparison with santanolide C, 38 (See Figure 10). The structure of the remainder of the compound was elucidated in a similar way to parthenolide (Part I, 3A) and the series of

reactions are summarised in Figure 10.

Santamarine has also been isolated from Ambrosia confertiflora by Yoshioka et al.[49]

C CHRYSARTEMINS A AND B

In 1969 Romo et al.[50] isolated two guianolides, chrysartemins A, 39, and B, 40, from C. parthenium. The structures of these



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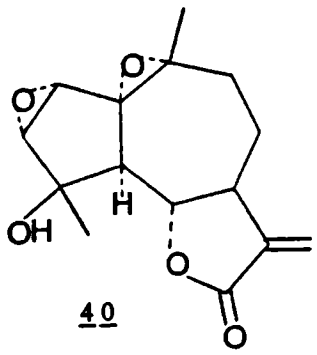


40

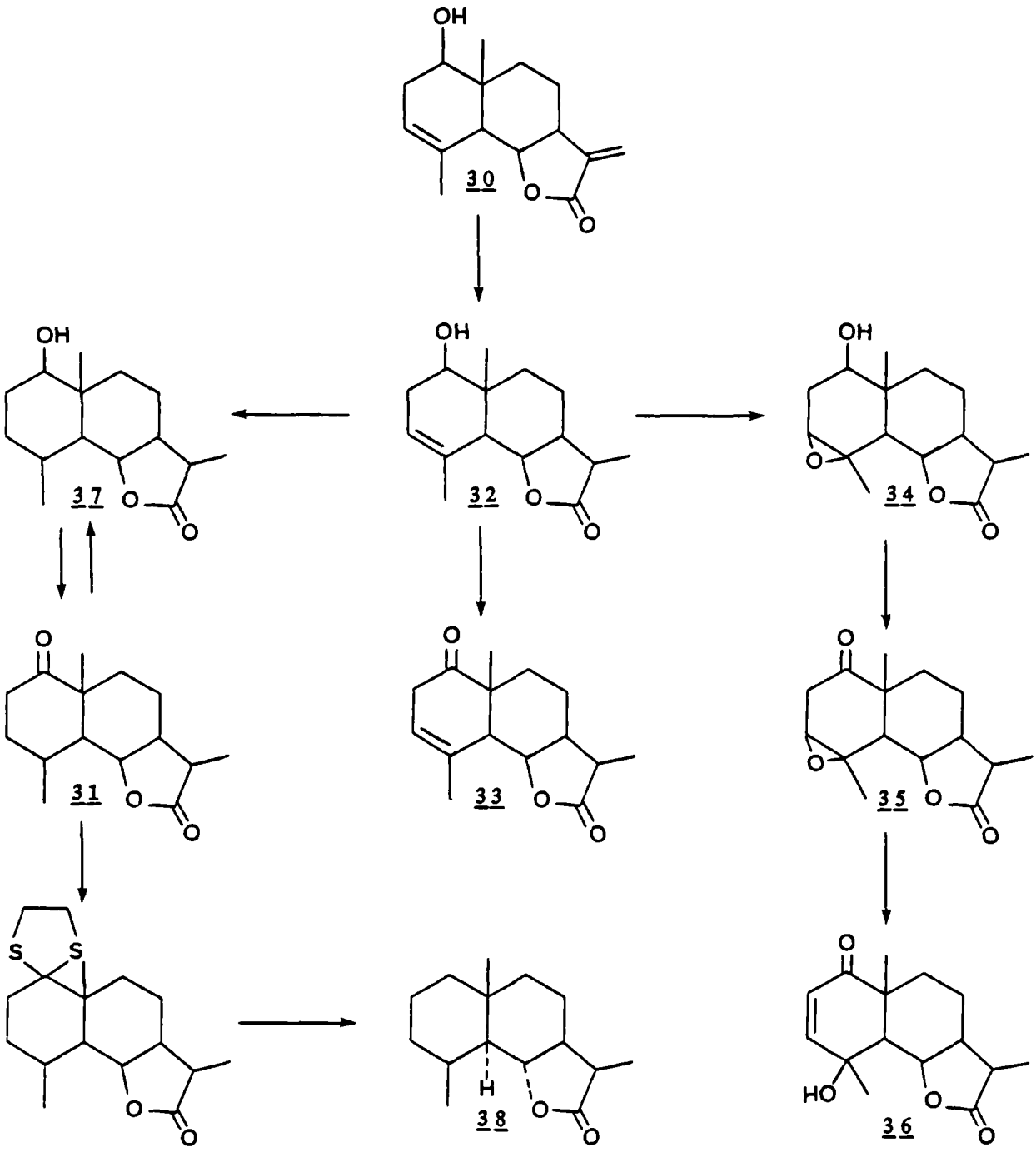
compounds were proposed mainly on evidence from infrared, nuclear magnetic resonance and mass spectrometry with additional chemical proof.

Chrysartemin A, 39, was shown to contain a hydroxyl group and an α-methylene-γ-lactone from infrared absorption bands. The hydroxyl group was assumed to be tertiary as it could not be acetylated and was resistant to chromic acid oxidation.

The NMR spectrum of 39 in DMSO-d_6 showed two doublets at δ5.96 and 5.50 (J = 3 Hz) for the exocyclic methylene hydrogens, a doublet of doublets at 4.47 (J = 11.5, 9.5 Hz) for the C-6 hydrogen, a doublet at 2.24 for the C-5 hydrogen and doublets (J = 1 Hz) at 3.42 and 3.25 for the hydrogens attached to the carbon atoms bearing the epoxide. The methyl group signals.

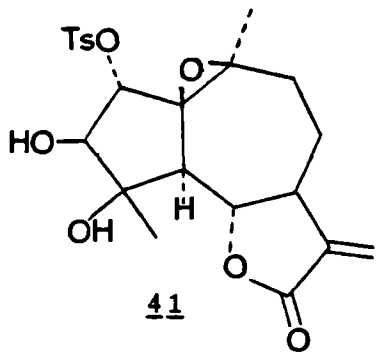
Figure 10

Summary of reactions leading to the structural elucidation of santamarine, 30



appeared as two singlets at δ1.38 (attached to a carbon atom bearing an ether oxygen) and 0.90 (attached to a carbon atom bearing a hydroxyl group). In $CDCl_3$, a signal at δ4.77 disappeared after equilibration with deuterium oxide and was assigned to the hydroxyl hydrogen. Hydrogenation of chrysartemin A gave the dihydro-derivative, the NMR spectrum of which showed a doublet (J = 7 Hz) in the methyl region. Aromatisation of chrysartemin A gave chamazulene and so a guiane structure was proposed with the lactone closed at C-6. The signal in the NMR for H-6 at δ4.47 is characteristic of a C-6 lactone in the guianolide series of sesquiterpene lactones.

On treatment with p-toluenesulphonic acid chrysartemin A gave the p-toluenesulphonate, 41, by opening of an epoxide the oxygen atom of which was borne on secondary carbons. This was shown in the NMR spectrum of the crude product by the appearance of two doublets at δ4.78 and 3.92. However, 41 also contained an epoxide formed between saturated carbons. Further spectroscopic studies confirmed the latter's position and hence the complete structure of chrysartemin A.

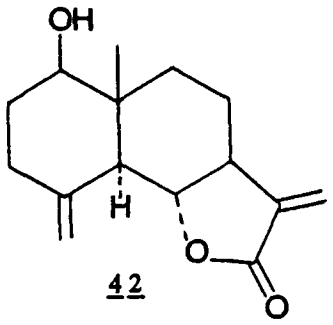


41

The structure of chrysartemin B, 40, was proposed by its very similar spectral properties to chrysartemin A, 39. Chrysartemin A has also been found in Artemisia mexicana and A. klotzchiana[50]

while both chrysartemins A and B have been found in Chrysanthemum morifolium[51] where they have been shown to stimulate root initiation.

Romo et al. also isolated santamarine, 30,[50] from C. parthenium and after crystallisation of this were able to isolate a minor constituent from the mother liquors. This was shown to be the eudesmanolide, reynosin, 42.



42

The infrared spectrum of reynosin, 42, showed absorption bands at 3520 and 3610 (hydroxyl), 1770 (γ-lactone) and 1680 cm^{-1} (conjugated double bond).

The NMR spectrum showed a pair of doublets (J = 3 Hz) at δ6.10 and 5.46 (exocyclic methylene conjugated with the γ-lactone), two broad singlets with long-range coupling at 5.01 and 4.89 (C-4 exocyclic methylene), an apparent triplet (J = 11 Hz) at 4.06 (H-6), a doublet of doublets at 3.53 (H-1), a sharp signal at 2.04 which disappeared on addition of deuterium oxide (hydroxyl hydrogen) and a methyl singlet at 0.85.

Reynosin has also been isolated from Ambrosia confertiflora.[49]

4 STRUCTURAL ELUCIDATION OF SESQUITERPENE LACTONES

A NMR SPECTROSCOPY

With the advent of NMR spectroscopy in the 1960s[52] the structures of many sesquiterpenes have been elucidated and the number of new structures appearing is increasing all the time. The presence of certain structural groups gives rise to characteristic spectral features which may be of particular help in the elucidation of these compounds such as an 11(13)-double bond, 8α-hydroxyl or ester group, long range couplings between H-6 and H-11 and between the C-5 methyl and the C-4 methylene hydrogens and the presence of C-6 or C-8 lactones.

(a) 11,(13)-double bond

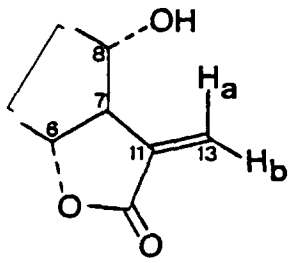
It is well established that allylic coupling occurs between the two C-13 methylene hydrogens and H-7 for all sesquiterpenes containing either a C-6 or C-8 α,β-unsaturated γ-lactone.[53,54] The signals for the C-13 hydrogens thus appear as two doublets (J = 1-4 Hz) between δ5.0 and δ6.5 p.p.m. However, in some sesquiterpenes containing both a C-6 α,β-unsaturated-γ-lactone and an 8α-hydroxyl group each of the C-13 hydrogens gives rise to a doublet of doublets as a result of geminal coupling in addition to the allylic coupling[55] (see partial structure in Figure 11). In 11(13)-unsaturated lactones not containing an 8α-oxygen function the H-13 geminal coupling is 0.5 Hz or less and so is not usually observed.[53,54]

Geminal coupling (J = 1 Hz or more) is thus only observed in the NMR spectra when both an 8α-hydroxyl group and a C-6 α,β-unsaturated-γ-lactones are present. Examination of

these spectra reveals that the signal for H-13a, i.e. the hydrogen trans to the γ-lactone carbonyl, always appears at lower field than in the spectra of corresponding compounds which differ only in that they do not contain the 8α-hydroxyl group and thus do not show the geminal splitting pattern.

Figure 11

Partial structure of a sesquiterpene lactone containing a C-6 α,β-unsaturated-γ-lactone and an 8α-hydroxyl group.



It has been proposed that the geminal coupling and the paramagnetic shift for H-13a results mainly from the van der Waals effect of the 8α-hydroxyl group upon the bonding orbital of H-13a.[56] Zurcher[56] calculated the ranges in ppm for the van der Waals paramagnetic shifts and these are compatible with those found experimentally (Tables 3 and 4).

This paramagnetic shift and the geminal coupling data can be used for various stereochemical assignments.[55] A positive shift in the range 0.4 - 0.7 ppm relative to the chemical shift for H-13a in a compound without an 8-hydroxyl group together with a geminal coupling for H-13a denotes an α-orientation for the 8-hydroxyl group and no such shift a β-orientation.

When the absolute stereochemistry is known a positive paramagnetic shift can be used for conformational analysis for example a 0.5 - 0.7 ppm shift for H-13a in germacranolides such as salonitenolide, 43, requires a distance of 2.0

Table 3

Calculated H-13a paramagnetic shifts based on different distances between H-13a and C-8 oxygen atoms

Distance Å	Calculated shift in ppm
2.0 - 2.5	0.2 - 0.6
3.0	0.0 - 0.1
3.5 - 4.5	0.0

- 2.5 Å between the 8α-oxygen atom and H-13a (see Table 4) and this distance is possible only when the conformation with regard to C-6, C-7 and C-8 is as shown in Figure 12. The assignment of the conformation at these positions usually permits the assignment of the conformation of the whole molecule.[31c]

(b) Long range couplings

(i) H-6 - H-11 couplings

The NMR spectra of 11,13-dihydro-eudesmanolides often show a broadened triplet for the C-6 lactonic hydrogen e.g. in colartin, 44,[31d] from Artemisia tripartita subsp. arbuscula

Table 4

Observed H-13a paramagnetic shifts and measured distances between H-13a and C-8 oxygen atoms using probable conformations of some sesquiterpene lactones

Type of C-6 α-lactone	$J_{13a,b}$ Hz	Distance 1 Å	Distance 2 Å	Shift in ppm	C-8 Conformation
8α-hydroxy					
eudesmanolides	0.7 - 1.2	2.0 - 2.5	*	0.4 - 0.6	equatorial
germacranolides	1.6 - 2.2	2.0 - 2.5	3.5 - 4.0	0.4 - 0.6**	equatorial
guianolides	0.7 - 1.8	2.0 - 2.5	3.5 - 4.0	0.4 - 0.6**	equatorial
8β-hydroxy					
eudesmanolides	0	*	3.5 - 4.0	0.0 - 0.1***	axial
germacranolides	0	2.0 - 2.5	3.5 - 4.0	0.0 - 0.1***	axial

Distance 1 - distance between H-13a and 8-oxygen atom in an equatorial conformation

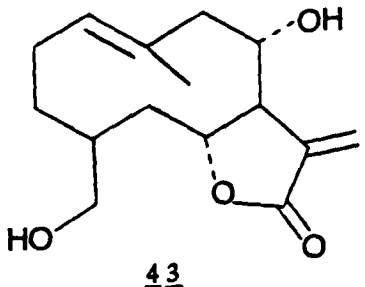
Distance 2 - distance between H-13a and 8-oxygen atom in an axial conformation

* - impossible conformation

** - observed shift supports equatorial orientation of 8α-hydroxyl groups

*** - observed shift supports axial orientation of 8β-hydroxyl groups

and Saussurea lactone, 45,[31e] from Saussurea lappa.



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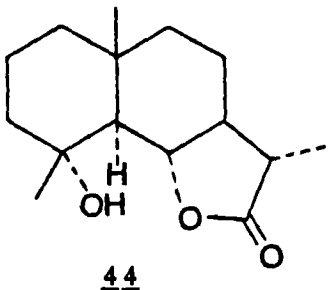

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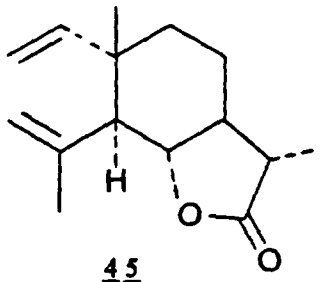

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(ii) Couplings between C-5 methyl and C-4 methylene hydrogens of 3,4-seco-pseudoguianolides

NMR spin decoupling experiments show that one of the C-4 methylene hydrogens of psilotropin, 46, couples with the 5-methyl group.[31f]

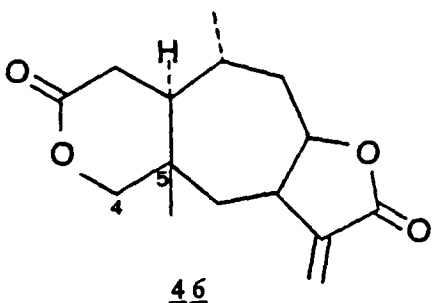

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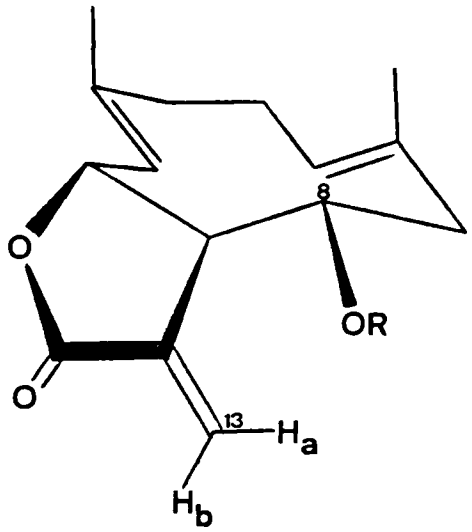
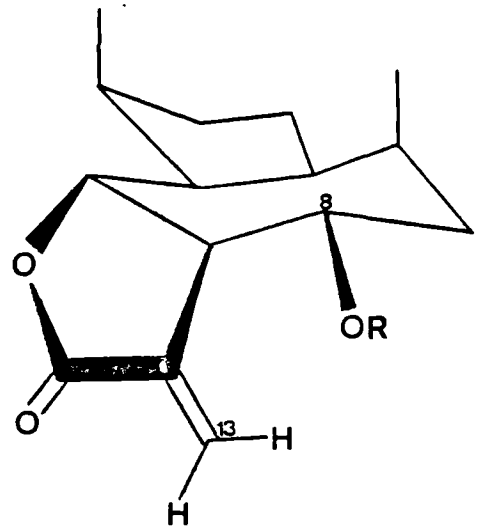
Figure 12

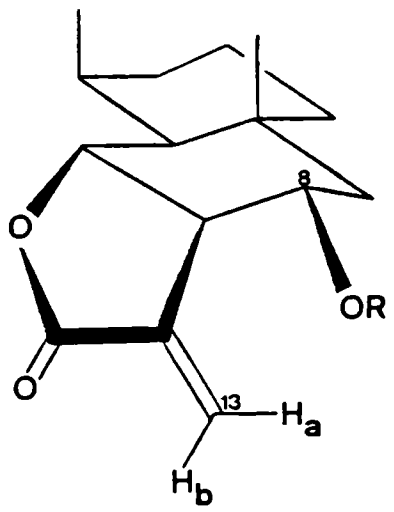
Conformation at C-6, C-7 and C-8 based on the distance between the 8α-oxygen atom and H-13a as required to account for the paramagnetic shifts in 6α-lactonised 8α-hydroxy sesquiterpene lactones



germacranolides

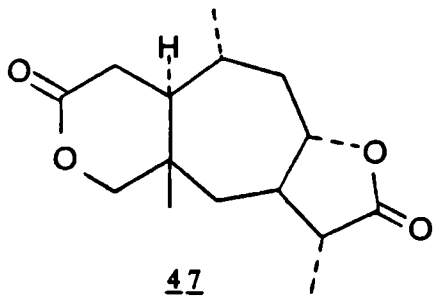


guianolides



eudesmanolides

Dihydrovermeerin, 47, exhibits a similar coupling in its NMR spectrum.[31g]



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(c) Trimethylsilyl ethers

Sesquiterpene lactones containing hydroxyl groups are often poorly soluble in non-polar solvents such as $CDCl_3$, CCl_4 and deuterated benzene. The acetyl analogues usually have better solubilities in these solvents but important signals associated with the sesquiterpene lactone skeleton often overlap. Thus the NMR spectra of hydroxy sesquiterpene lactones are often best determined as the trimethylsilyl ethers. As with the steroids and tetra- and pentacyclic triterpenes, hydroxylated sesquiterpenes readily form relatively volatile trimethylsilyl ethers which are readily soluble in non-polar solvents and give good results where high resolution is required.[57]

Recently however high performance liquid chromatographic separations of underivatised sesquiterpenes are beginning to supercede this technique.

(d) Nuclear Overhauser effects and variable temperature studies

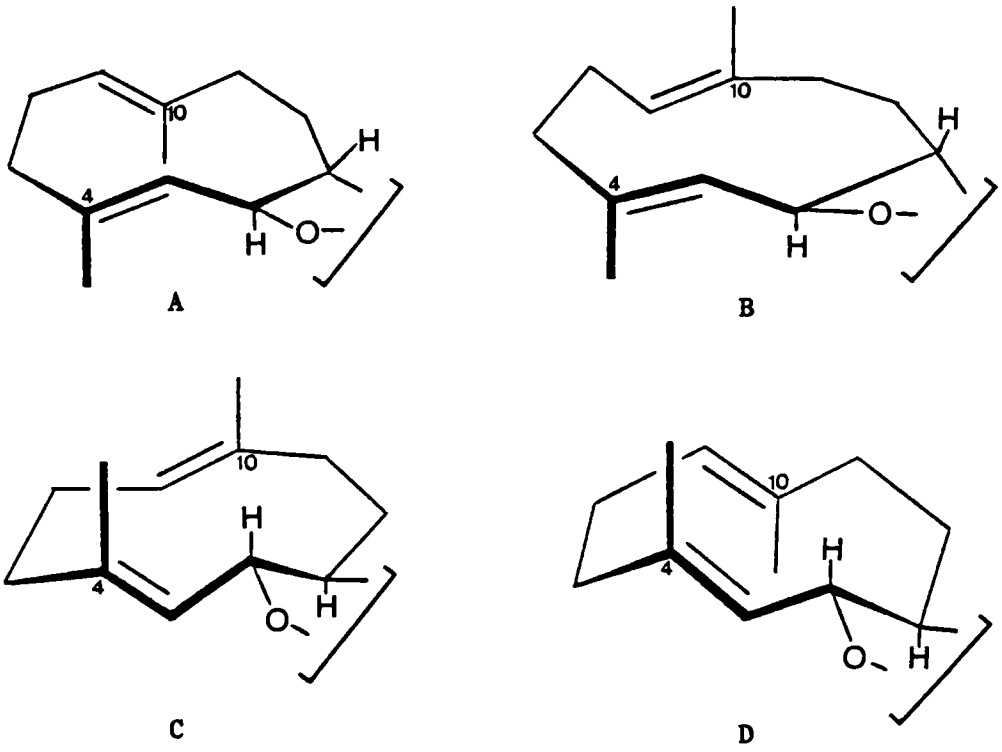
Germacranolides may exist in different conformational forms in solution.[58] Nuclear Overhauser effect[59a] analyses and NMR spectral studies at different temperatures[60] can often

distinguish conformers although no absolute methods are available.

Germacranolides exist in four major conformational forms as shown in Figure 13.[31h]

Figure 13

Four major conformational forms of germacranolides



The compound dihydrotamaulipin A acetate, 48,[61] was shown to have the conformation 49 shown by NOE techniques (Figure 14).

With reference to 49, an increase in the integrated intensity of the H-2 and H-6 signals caused by irradiation of the C-4 methyl signal, as well as enhancement of the H-2 signal by irradiation of the C-10 methyl signal, indicated

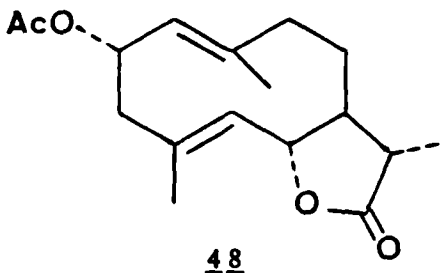


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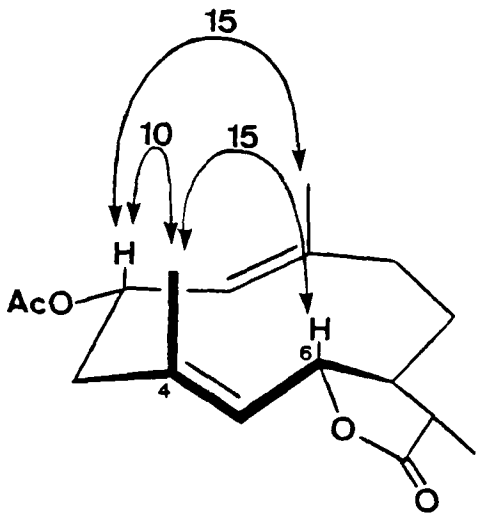
Figure 14

Conformations of dihydrotamaulipin A acetate. The figures indicate percentage NOE enhancements.

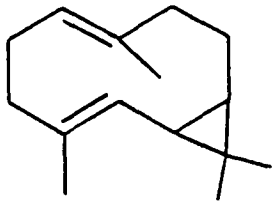


49

that the 10-membered ring adopted the conformation shown i.e. in which the C-4 methyl, C-10 methyl, H-2 and H-6 are in the same direction. This corresponds with conformation C in Figure 13.

The conformations of furanodienone,[62] linderalactone,[63] bicyclogermacrene, 50, and of iso-bicyclogermacrene, 51,[64] have been determined by similar techniques.

These latter two are of interest because it is postulated that they are the biogenetic precursors of other sesquiterpenes containing a fused 1,1'-dimethylcyclopropane ring.[64] Bicyclogermacrene was shown to adopt conformation A



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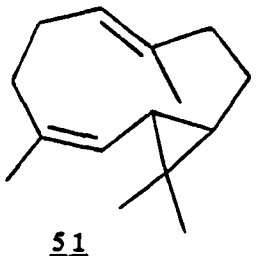

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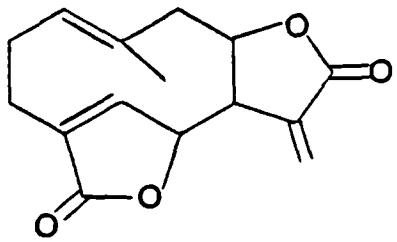
and iso-bicyclogermacrene C in Figure 13.

Isabelin, 52, is a naturally occurring germacranolide isolated from Ambrosia psilostachya.[60] NMR studies at different temperatures established that isabelin existed in



52

solution at room temperature in a 10:7 ratio of two conformers. The NMR spectrum recorded in $CDCl_3$ at 25° showed two sets of signals which appeared to correspond with two compounds in a 10:7 ratio. The material behaved as a single compound chromatographically, during fractional crystallisation experiments as well as during the preparation of a number of derivatives and transformation

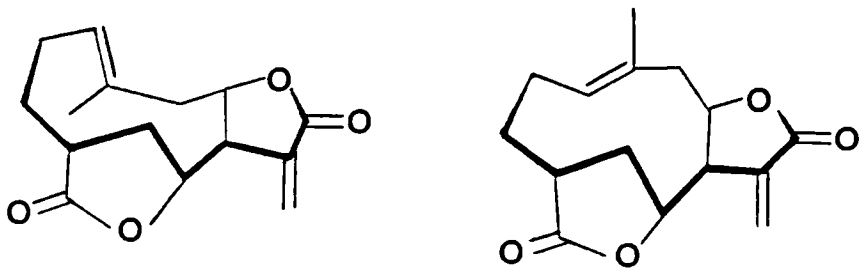
products. These results suggested that the NMR spectrum of isabelin should be interpreted on the basis that isabelin exists as two conformational isomers in solution at room temperature. Conclusive evidence for this was provided by a temperature controlled NMR study. Crystalline isabelin was dissolved in $CDCl_3$ precooled to -50° and the NMR spectrum recorded at this temperature within 40 minutes. The major isomer appeared to correspond to the minor form at 25° and this is probably the only conformer present in the crystals. When the solution used for this $-50^{\circ}C$ NMR study was allowed to warm to room temperature it again showed the 10:7 ratio of conformers.

Two conformational forms A and B of isabelin were proposed from consideration of molecular models and the different values observed for their coupling constants (Figure 15).[60] The bond angle between H-7 and H-6 in conformer A is approximately 90°, a value in accord with a small coupling constant.[65a] The major form of isabelin in solution at room temperature showed a 1 Hz coupling and was therefore assigned structure A. The bond angle between H-7 and H-6 in B is approximately 180° and thus a larger coupling constant is expected. The minor conformer of isabelin exhibited a 7 Hz coupling constant and was therefore assigned structure B.

These two conformations were confirmed in 1971 by K. Tori et al.[66] by nuclear Overhauser effect experiments. They also proved the endocyclic double bond to have a trans orientation, and since isabelin has been correlated with artemisiifolin, 53, salonitenolide, 43 and cnicin, 54, the results obtained also established a trans configuration for

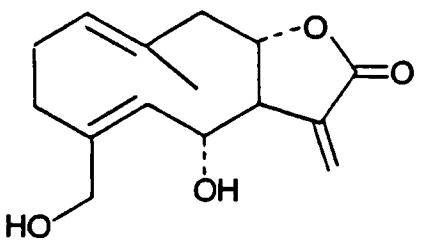
Figure 15

Two conformational forms of isabelin



A B

these other germacranolide monolactones.



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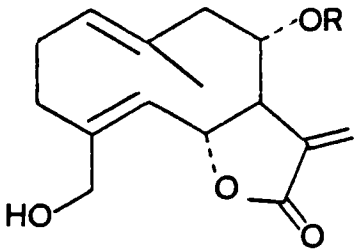

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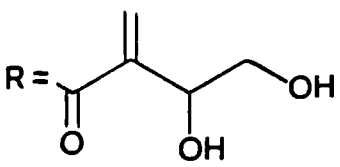


54

Similarly laurenobiolide exists as two conformers at low temperatures in the ratio 8:2.[67] At temperatures higher than 100°C, the NMR spectrum of laurenobiolide showed one

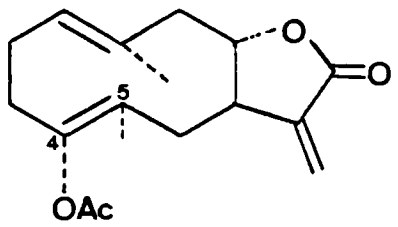
set of sharp signals indicating a rapidly inverting ten-membered ring while at temperatures lower than -20°C, two sets of signals were observed, one for each isomer.

The conformations of neolinderalactone having cis-1(10) and trans-4(5) double bonds and sericenine having trans-1(10) and cis-4(5) double bonds were also studied by intramolecular NOE and variable temperature studies.[68]

Two crystalline compounds urospermal A and B are conformational isomers which have been shown to be interconvertible in solution.[69] Each conformer is considered to be stabilised by intramolecular hydrogen bonding and the two forms are separable by chromatography.

B X-RAY DIFFRACTION METHODS

In addition to nuclear Overhauser effect studies, X-ray diffraction methods have been useful in the elucidation of structures and investigation of conformers in sesquiterpene lactones such as parthenolide, 20,[45] and scorpioidine, 55.[70]



55

The unusual cis (with respect to the C-chain) 4,5 double bond in 55 was confirmed by X-ray analysis as was the trans-fused 7-8 bond. Most sesquiterpenes are trans-fused across the 6-7 bond.

5 BIOLOGICAL ACTIVITY IN THE FAMILY COMPOSITAE

The Compositae is one of the largest families in the plant kingdom comprising 1000 genera and 15000 species but, until recently, has been the source of relatively few products of medicinal and economic importance. Only about 30 species are used as crude drugs with just 16 drugs appearing in the pharmacopoeias. No more than 20 pure substances are used therapeutically or available commercially.[71a] The current interest in the family stems largely from improved structural elucidation techniques, which has led to the large number of novel sesquiterpenes found together with the use of new screening methods and of computer evaluation.

Seven main types of biological activity have been shown to be present in the family namely, A cytotoxic, B spasmoloytic, C anti-inflammatory, D antihepatoxic and cholerectic, E antimicrobial, F antihyperlipidemic and G insecticidal.

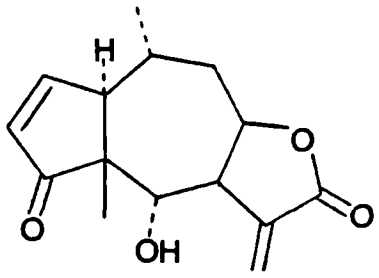
A CYTOTOXIC ACTIVITY

The discovery of new compounds with cytotoxic activity has received much publicity in recent years in view of their possible use in cancer. The availability of modern, refined methods of testing antitumour agents has encouraged a systematic search for these agents from natural sources. Not surprisingly, therefore, perhaps the greatest amount of work with regards to biological activity in the Compositae has been directed towards this aim. Many of the sesquiterpene lactones found in the family, chiefly the germacranolides, guianolides and elemanolides, are especially active.[71b] The first positive results were obtained with chamomile extracts and chamazulene[72] in the 1950s but more recent examples include parthenolide, 20,[73]

helenalin, 56,[73] paucin, 57,[73] molephantinin, 58,[74,75] eupahyssopin, 59,[76] cnicin, 54,[77] chlorohyssopifolin C, 60,[78] microlenin acetate, 61,[79] rudmollin, 62,[80] and piptocarphin A, 63.[81]

In 1943 Medawar et al.[82] suggested that the cytotoxicity of cardenolides and related compounds was associated with the presence of the unsaturated lactone. Subsequent studies seem to suggest that the antitumour activity of sesquiterpene lactones is due to the presence of an α-methylene group on the γ-lactone ring as well as another functional group such as an epoxide, chlorhydrin, unsaturated ester, unsaturated lactone or an unsaturated ketone.[73,83,84] Little is known however of the relation between structure and activity in these compounds. Nevertheless the demonstrated reactivity of unsaturated lactones towards thiols and amines and the presence of other reactive functional groups suggest that the cytotoxicity may result from irreversible alkylation of nucleophilic centres in a biological system[73,83,84] such as the Michael-type addition of α-methylene-γ-lactones with cysteine (Figure 16).

Hladon and Twardowski[84] have investigated the mode of action of some sesquiterpene lactones at a cellular level using HeLa cells. At subtoxic concentrations they demonstrated arrest of the HeLa cell in interphase (G_1 and/or S, G_2) and, at higher concentrations complete and irreversible cytotoxic effects with pyknosis (chromosome condensation leading to degeneration of cell nuclei) and karyorrhexis (breaking of cell nuclei, disintegration of chromatin into shapeless granularity). At the molecular level they found inhibition of protein and RNA synthesis and the translation process, but no significant



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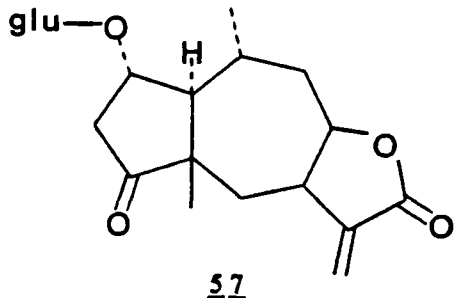

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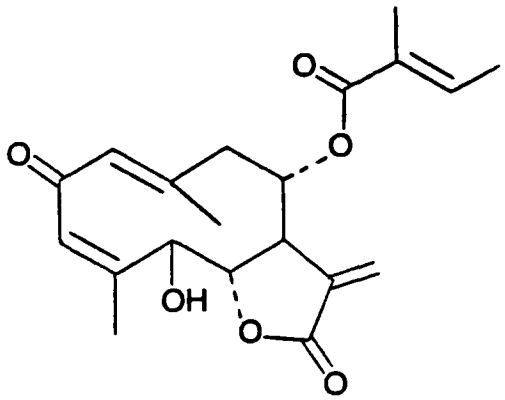

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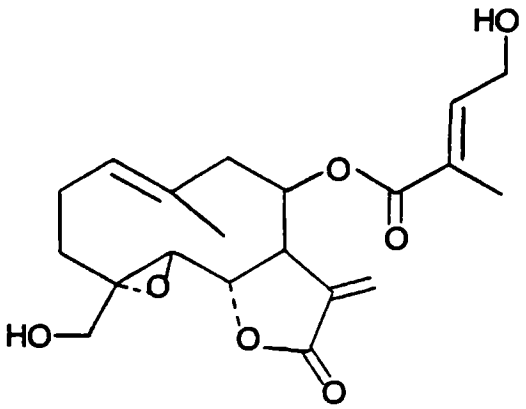

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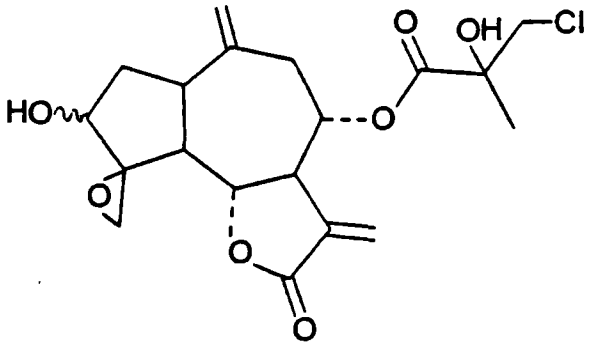

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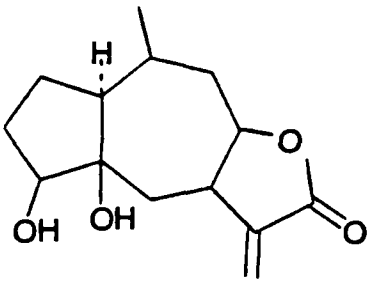

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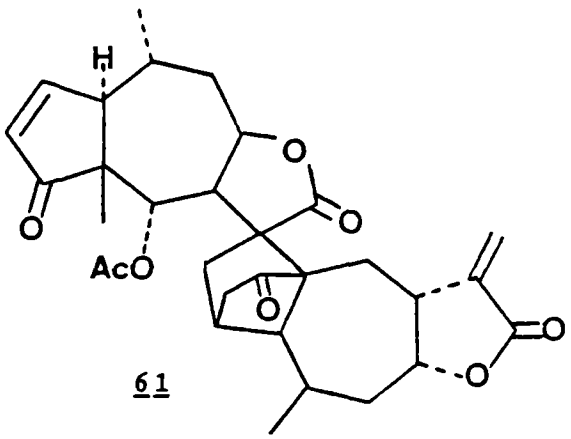

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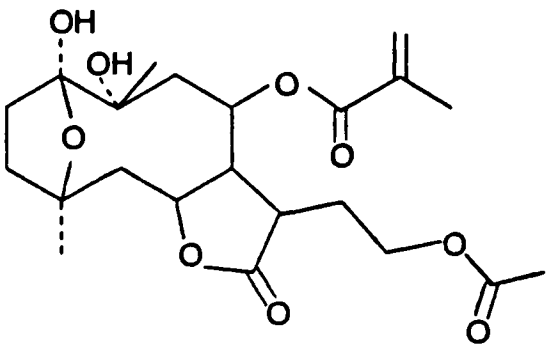
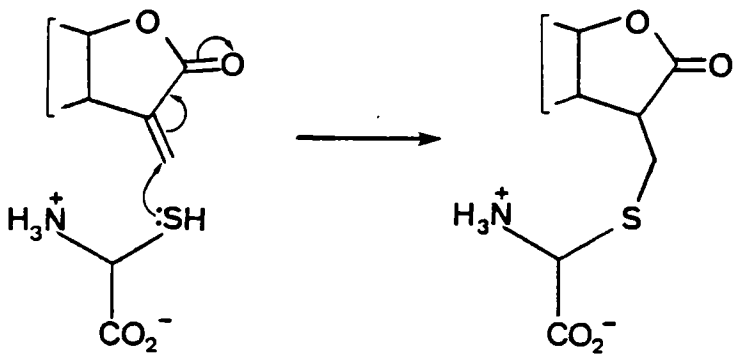


63

inhibition of DNA synthesis. These results led them to propose a hypothetical model of the cytostatic action of sesquiterpene lactones (Figure 17).

Figure 16

Michael-type addition of an α-methylene-γ-lactone with cysteine.



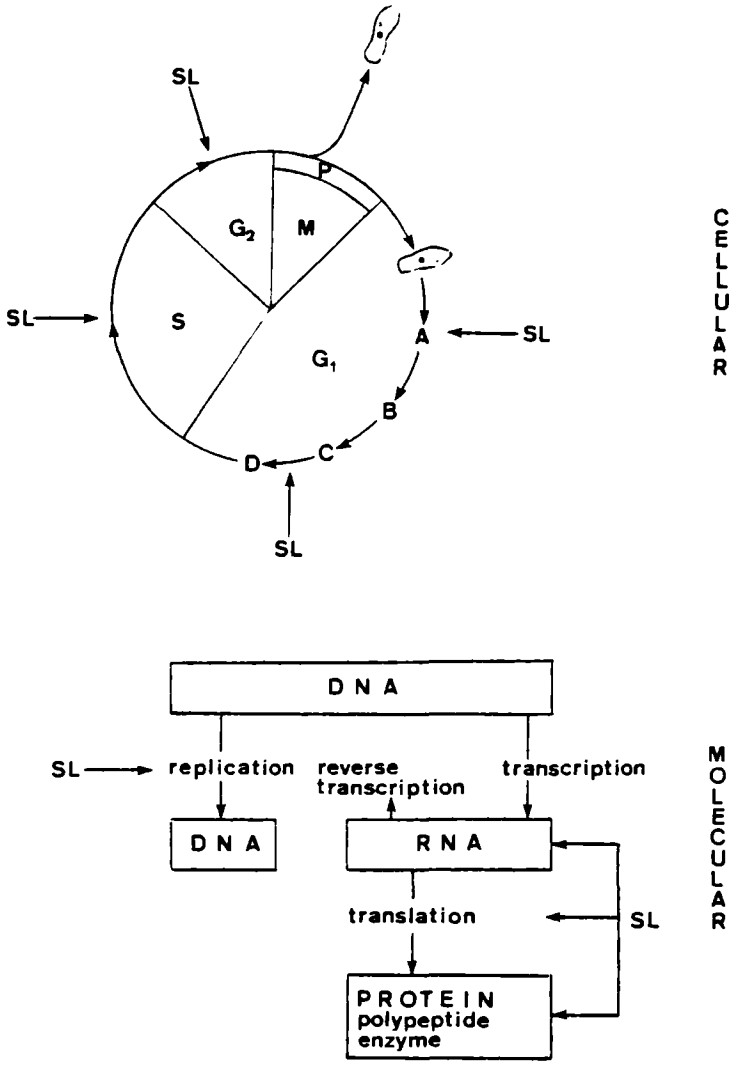
The therapeutic use of cytotoxic sesquiterpenes has been prevented by their relatively high toxicity but chemical modification may result in an increased therapeutic index.[85,86] The isolation of new compounds with cytotoxicity to use as tools to interpret the biochemical mechanisms involved in tumour growth and control is also of great importance.

B SPASMOLYTIC ACTIVITY

As early as the Middle Ages, the leaves and roots of Petasites hybridus were used for their anticonvulsive activity in asthma[87] and in disturbances of the alimentary canal but it was not until the late 1950s that the active principles were identified. They were the compounds petasin, 64, iso-petasin, 65, S-petasin, 66, and S-iso-petasin, 87.

Figure 17

Hypothetical model of cytostatic action of sesquiterpene lactones

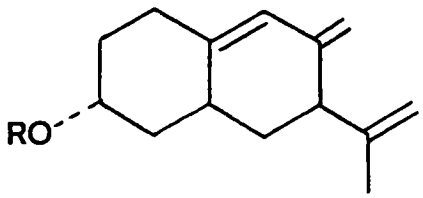


SL sesquiterpene lactone

These compounds belong to the eremophilane class and are esters of the 15-alcohol petasol or iso-petasol with angelic acid or methylmercaptoacrylic acid respectively.

Unfortunately Aebi et al.[87] did not specify the method used in

assessing spasmolytic activity. However, they did find that chromatography on alumina destroyed activity probably by a ring opening reaction under the basic conditions. The activity was unchanged after chromatography on silica gel.



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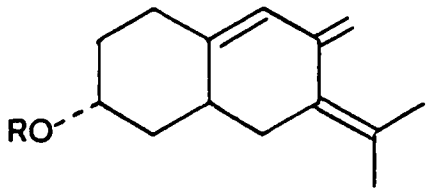

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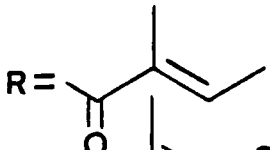

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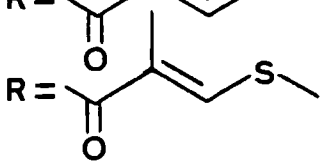

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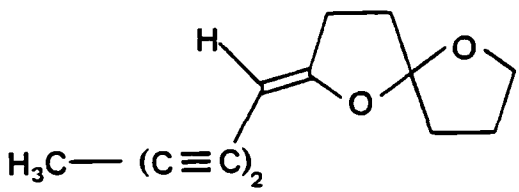
Matricaria chamomilla has also been shown to possess spasmolytic activity but here the activity resides in the flavone glycosides for example apigenin-7-glycoside and cis-spiroether, 68.[71a,88,89]



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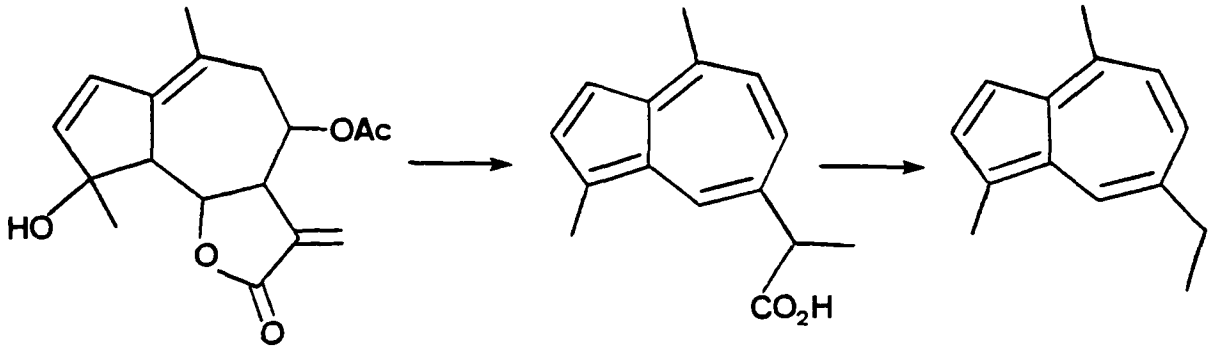
C ANTIINFLAMMATORY ACTIVITY

The use of Matricaria chamomilla is well known for its anti-

inflammatory activity.[71a] For a long time the only known active principle was the blue azulene compound chamazulene, which is produced from matricin, 69, a guianolide, during steam distillation (Figure 18).

Figure 18

Production of chamazulene during steam distillation of chamomile oil



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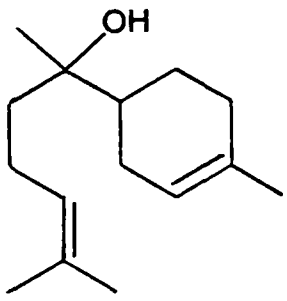
More recently however other more active compounds have been found such as α-bisabolol, 71, an unsaturated monocyclic sesquiterpene alcohol.



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Bisabolol ethers and esters have been prepared by semi-synthetic routes to give compounds with enhanced antiphlogistic activity.[90] Spiroether, which is abundant in chamomile and

possesses spasmolytic activity (Part I, 5B) has also been shown to have antiphlogistic activity.[71c]

Hall et al.[91] have subsequently studied the mode of action of sesquiterpene lactones as antiinflammatory agents and have found, as with cytotoxic activity, the presence of an α-methylene-γ-lactone moiety to be of importance. Compounds containing this grouping were shown to be potent inhibitors of carrageenan-induced oedema and chronic adjuvant-induced arthritis in rodents. Helenalin, 56, was found to be the most potent antiinflammatory agent of the 22 compounds tested.

In the carrageenan-induced oedema screen, the presence of an α-epoxycyclopentanone system in addition to the α-methylene-γ-lactone contributed to activity whereas in the adjuvant-induced arthritis, a third grouping, i.e. a β-unsubstituted cyclopentenone ring, also instilled antiarthritic activity.

Hall et al.[91] concluded that sesquiterpene lactones appeared to be similar in activity to commercially available agents such as indomethacin, due to their inhibition of neutrophil migration, lysosomal rupture, enzymatic activity and prostaglandin synthesis. In addition, only the germacranolides tested did not elevate cyclic adenosine monophosphate levels.

D ANTIHEPATOXIC AND CHOLERECTIC ACTIVITY

The increased incidence of liver diseases in the western world is due mainly to increased alcohol consumption and bad diet. The development of liver protection agents or substances that increase the ability of the liver to regenerate is therefore of great importance.[71d,92a]

The fruits of Silybum marianum have been used as a liver remedy since Dioscorides in A.D.50.[6c] In 1949 Eichler and Hahn and Mayer and Merge[71e] reported that a tincture of the drug gave protection to the liver against trinitrotoluene and carbon tetrachloride and was successful against hepatitis. In 1968 Hahn et al.[93] found that the active principles were in the flavonoid fraction of the drug. The flavonoids silybin, silydianin and silychristin were isolated and shown to be the active constituents.[94]

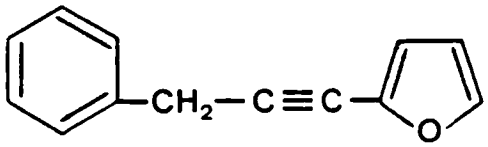
In these compounds the flavone molecule is attached to coniferyl alcohol. They were given the collective name silymarins. One site of action of silymarins is the outer cell membrane of the liver where for example silybin can block the attachment of a poison such as phalloidin to specific membrane receptors. It is also the only known compound capable of displacing phalloidin after it has become bound to a liver cell. Silybin also stimulates the synthesis of ribosomal RNA in the nuclei of hepatocytes.[71f]

Helichrysum arenarium has also been used in folk medicine as an anticholerectic and remedy of liver diseases. The naringenin glycosides helichrysin and salipurposide are thought to be responsible for its action.[71f]

Cynarin from the artichoke, Cynara scolymus, causes an increase in bile secretion and this is thought to be primarily responsible for its cholerectic and cholagogic activity. The aqueous leaf extracts also cause an increase in the number of binucleate hepatocytes and in the RNA concentration of liver cells.[71g]

E ANTIMICROBIAL ACTIVITY

Many polyacetylene compounds found in the family Compositae possess bacteriostatic or fungistatic properties for example Carlina oxide, 72, from Arlina acaulis,[95] capillin, 73, from Artemesia capillus,[96] trideca-1-monoene-3,5,7,9,11-pentayne, 74, and trideca-1,11-diene-3,5,7,9-tetrayne, 75, from Arnica montana, Arctium lappa and species of Echinacea and Pulicaria.[96]



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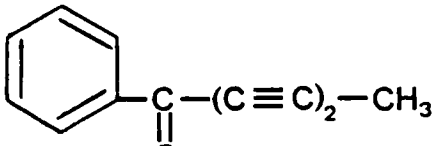

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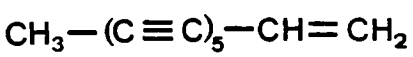

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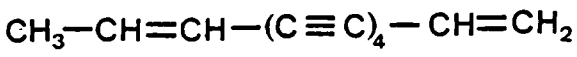

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The therapeutic use of these compounds, however, is limited by their instability and high toxicity. Many synthetic analogues were therefore prepared[97] in the hope of increasing stability and decreasing toxicity. Systematic microbiological investigations of the natural and synthetic polyacetylenes provided much information about structure activity relationships. If a non-terminal triple bond is present then one substituent should be an aromatic residue and the other should carry a functional group such as an ester, thioamide, carbonyl, hydroxyl, aldehyde, halogen, ethylene or acetylene adjacent to the triple bond. When a terminal triple bond is present, the substituent should be an

aromatic acyl residue. Compounds of weaker activity are obtained if this is replaced by an aliphatic residue. Fungistatic activity was found to increase with the polarisation of the triple bond and with lipid solubility whereas bactericidal activity increased with the hydrophilic nature of the compound.[97]

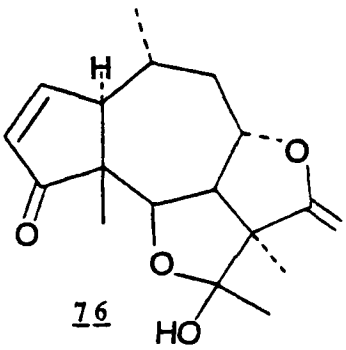
Antimicrobial activity in the family Compositae is not confined to the polyacetylenes. In 1979 Blakeman and Atkinson[98] showed that parthenolide, 20, inhibited the growth of Gram-positive bacteria, yeasts and filamentous fungi. On the basis of chromatograms of extracts of Chrysanthemum parthenium they suggested that parthenolide is located in the glands on the surfaces of the leaves and seeds. This confirms the work of Loomis and Croteau[99] who have stated that accumulation of sesquiterpenes in large quantities in plants is almost always associated with the presence of glandular structures. In addition Blakeman and Atkinson[98] found at least four other components present in the crude chloroform extracts of leaves and seeds of C. parthenium to possess antimicrobial activity. They also postulate that a possible function of the glands may be protection of the plant against pathogens.

Bacteriostatic activity is also exhibited by the many phenolic carboxylic acids that occur in the Compositae, such as caffeic acid and chlorogenic acid. Echinacea preparations have been used pharmaceutically as bacteriostats.[100] Part of this activity resides in a complex depside consisting of dihydroxyphenyl-ethanol, caffeic acid, rhamnose and 2 molecules of glucose.[101] Echinacea total extracts also show antiviral activity[102] although the active components have not yet been

isolated.

F ANTIHYPERLIPIDEMIC ACTIVITY

Hall *et al.*[103] have reported that some naturally occurring guianolides and germacranolides as well as synthetic related compounds are antihyperlipidemic agents in mice. Several of the compounds tested, for example helenalin, 56, tenulin, 76, 2,3-epoxytenulin, 77, deoxyelephantopin, 78, and eupahysopin, 59, at a daily dose of 20 mg/kg resulted in lowering of serum



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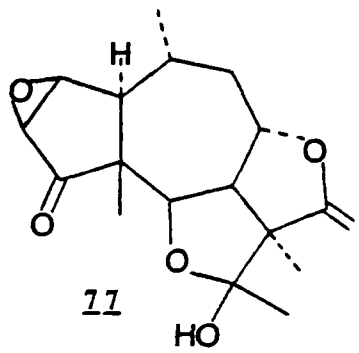

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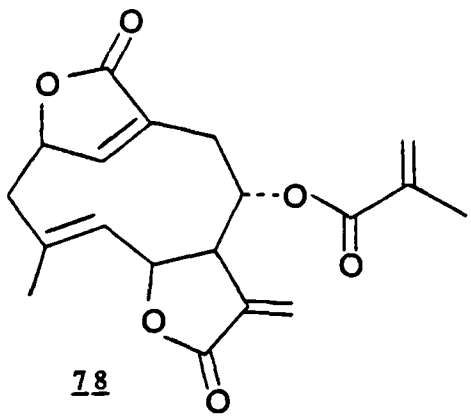

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cholesterol by more than 30% and serum triglycerides by 25%. The commercially available compound Clofibrate had no effect at 20 mg/kg and required a daily dose of 300 mg/kg in rodents to reduce serum cholesterol by 23%. The sesquiterpene lactones therefore warrant further investigation as antihyperlipidemic

agents.

Certain features in the molecule appeared to be responsible for lowering serum lipids including an α-methylene-γ-lactone, β-unsubstituted cyclopentenone ring and α-epoxycyclopentanone system. The compounds probably act by alkylating the thiol-bearing enzymes of lipid synthesis such as acetyl-CoA, citrate-lyase, acetyl CoA synthetase and β-hydroxy-β-methylglutaryl-CoA reductase by a Michael-type addition.[83]

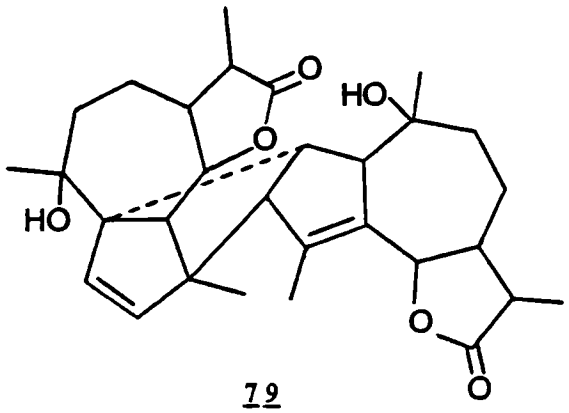
G INSECTICIDAL ACTIVITY

The insecticidal properties of Chrysanthemum cinerariaefolium have been known for a long time[104] and with the move against the use of DDT are of renewed interest. The insecticidal activity is due to the presence of six constituents: pyrethrins I and II, cinerins I and II and jasmolins I and II. They are esters of chrysanthemic acid (I series) or pyrethric acid (II series). For activity the acid moiety must contain a cyclopropane ring with gem-dimethyl groups. The free acids are inactive.[71h]

Much work has been done on structure activity relationships[71h] and this has led to the preparation of synthetic compounds such as allethrin which has about the same activity as pyrethrin I.

Other compounds with insecticidal activity have been isolated from species of Echinacea, Chrysanthemum, Heliopsis and Anacyclus.[71h] These are isobutylamides of long chain simple unsaturated fatty acids or acetylene fatty acids. They probably act as natural protective agents against insect attack in the plant.

Absinthin, 79, a dimeric sesquiterpene from Artemesia absinthium also has protective properties against insect attack.[105]



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6 MIGRAINE

The earliest description of migraine can be found in a Sumerian poem written about 300 B.C.[106] Later, Hippocrates described a syndrome of periodic headaches associated with visual disturbance and vomiting. He used the term hemicrania, i.e. affecting one side of the head. The present day Anglo-Saxon name migraine stems from the Old English megrine which was derived from the Latin migrane.[107]

The characteristic features of migraine are paroxysmal headache often but not invariably unilateral usually associated with visual disturbances and vomiting. It is one of the most common neurological disorders with an incidence in the region of 12% of the population.[92b,108-110]

There is an initial phase of vasoconstriction which is responsible for the visual symptoms or 'aura' followed by vasodilatation. These changes affect both the internal and external branches of the carotid artery. It is the dilatation of branches of the external carotid

artery and to a lesser extent the internal carotid artery which is associated with the pulsatile headache. The vasoconstriction is believed to be due to the release of amines such as noradrenaline from neurones, 5-hydroxytryptamine from platelets and histamine from mast cells.[92b,108-111]

The cause (or causes) of migraine is unknown but there is a genetic predisposition. Attacks are often related to some emotional disturbance and sometimes precipitated by eating foods rich in monoamines such as tyramine i.e. cheese.[92b,108-110]

In about one third of women migrainous symptoms are associated with the menstrual cycle and this may account for the increased incidence found in females.[92b,109,111] Ovarian steroids and oral contraceptives may interfere with catecholamine metabolism. In addition, 17β-oestradiol and progesterone have been shown to potentiate the effects of noradrenaline, adrenaline and tyramine.[111] Prostaglandins may also be involved in migraine since drugs inhibiting prostaglandin synthesis such as aspirin are effective for both prophylactic and acute treatment.

In the treatment of migraine, drugs with antinoradrenaline (e.g. ergot alkaloids), anti-5-hydroxytryptamine (e.g. ergot alkaloids, methylsergide, cyproheptidine) and antihistamine (e.g. prochlorperazine) activities are all inconsistently efficacious. The action of the ergot alkaloids probably depends upon the existing vascular tone. Where there is low vascular tone the drug acts predominantly as a constrictor agonist. This may be by action on adrenoreceptors or 5-hydroxytryptamine receptors. In the presence of marked neurogenic tone the α-adrenoreceptor blocking action of the drug is paramount and vasodilatation results. The action of these drugs in migraine

therefore depends upon the stage at which they are administered. In the constrictor stage the ergot alkaloids should cause dilation thereby preventing the consequences of the constriction since it is the initial vasoconstriction that appears to be responsible for the subsequent features of the migraine attack. This may explain why treatment with these drugs is more often successful when begun early in the prodromal phase. The vasoconstrictor action of the alkaloids can induce migraine if the vessels are not constricted at the time of medication.[111] The ergot alkaloids also have anti-5-hydroxytryptamine actions and it may be that a successful migraine treatment requires a combination of actions since the condition probably arises from more than one cause.

In looking for new drugs with potential use in migraine it seems reasonable therefore to test initially for spasmolytic activity in vitro. An initial screen for activity must use a preparation that responds well and gives rapid reproducible results. Such a preparation is the guinea pig ileum and appropriate agonists would be vasoactive substances such as acetylcholine, bradykinin, histamine, 5-hydroxytryptamine and noradrenaline, all, at one time or another, implicated in the pathogenesis of migraine.[112]

PART II

D I S C U S S I O N

Natural products have been and remain an important source of biologically active compounds. The current misconception in the medical profession as to the lack of value of these compounds has no grounding. In 1973, the last year for which figures are available, 25.2% of the prescriptions dispensed in the United States contained one or more constituents derived from higher plants.[113] The role of these compounds in the development of new semi-synthetic or synthetic drugs with enhanced efficacy or decreased toxicity cannot be underestimated. In addition the study of new compounds provides valuable pharmacological information and, together with synthetic derivatives, indications of possible structure-activity relationships. We should not forget that virtually every pharmacological drug prototype exhibiting the classical effects of the class concerned is derived from plant sources.

It is clear therefore that we must continue to look to plants as sources of new drugs. These sources remain largely untapped. If the main objective is to isolate constituents with pharmacological activity the procedure undertaken must be directed precisely towards this aim.

1 SCREENING PROCEDURE

Feverfew is a plant used for the alleviation of migraine. Since it is not possible to produce migraine in experimental animals a technique *in vitro* that could be related to the clinical situation had to be selected. The cause of migraine is probably broadly based so the initial screening for activity had to be such that false negative results could be reduced to a minimum. This also necessitated the use of a sensitive method since extracts prepared may have contained only very small quantities of active compounds.

The use of large quantities of material required for a less sensitive method would be impractical. The technique for evaluating biological activity had also to provide rapid reproducible results.

Guinea pig ileum is a preparation that responds well and is readily available. It was therefore selected for the primary screen. The agonists acetylcholine, 5-hydroxytryptamine and histamine were chosen to give a broad guide to general spasmolytic activity. Such activity would be useful in the treatment of migraine. The prostaglandin PGE_2 was also used as an agonist in selected cases.

The procedure consisted of recording the log (dose) vs response curves to acetylcholine, 5-hydroxytryptamine and histamine in an organ bath containing guinea pig ileum. ED_{50} doses were taken and given repeatedly until constant responses were achieved. The antagonist i.e. the extract or fraction obtained from column chromatography was then dissolved in the minimum of dimethylsulphoxide and made up to a concentration of 10^{-4} g/ml with Krebs solution. This was added to the bath containing the ileum and left for 30 minutes. The response of the tissue to the agonist was then recorded and the percentage change taken. A control experiment was performed in exactly the same way except that the antagonist was omitted.

Figures 19 and 20 show typical results obtained. Figure 19 shows sub-fractions A59-65 (see Part III, Table 19) caused 100% antagonism to all three agonists at 10^{-4} g/ml and the receptor sites of these agonists remained blocked even after washing with Krebs solution. The antagonism was therefore non-competitive. Figure 20 shows that sub-fractions A30-34 (see Part III, Table 19) caused approximately 50% antagonism to the agonists but this blockade was reversible after washing with Krebs solution, i.e. competitive antagonism.

Figure 19

Spasmolytic activity of sub-fractions A59-65 tested at 10^{-4} g/ml (see Part III, 3)

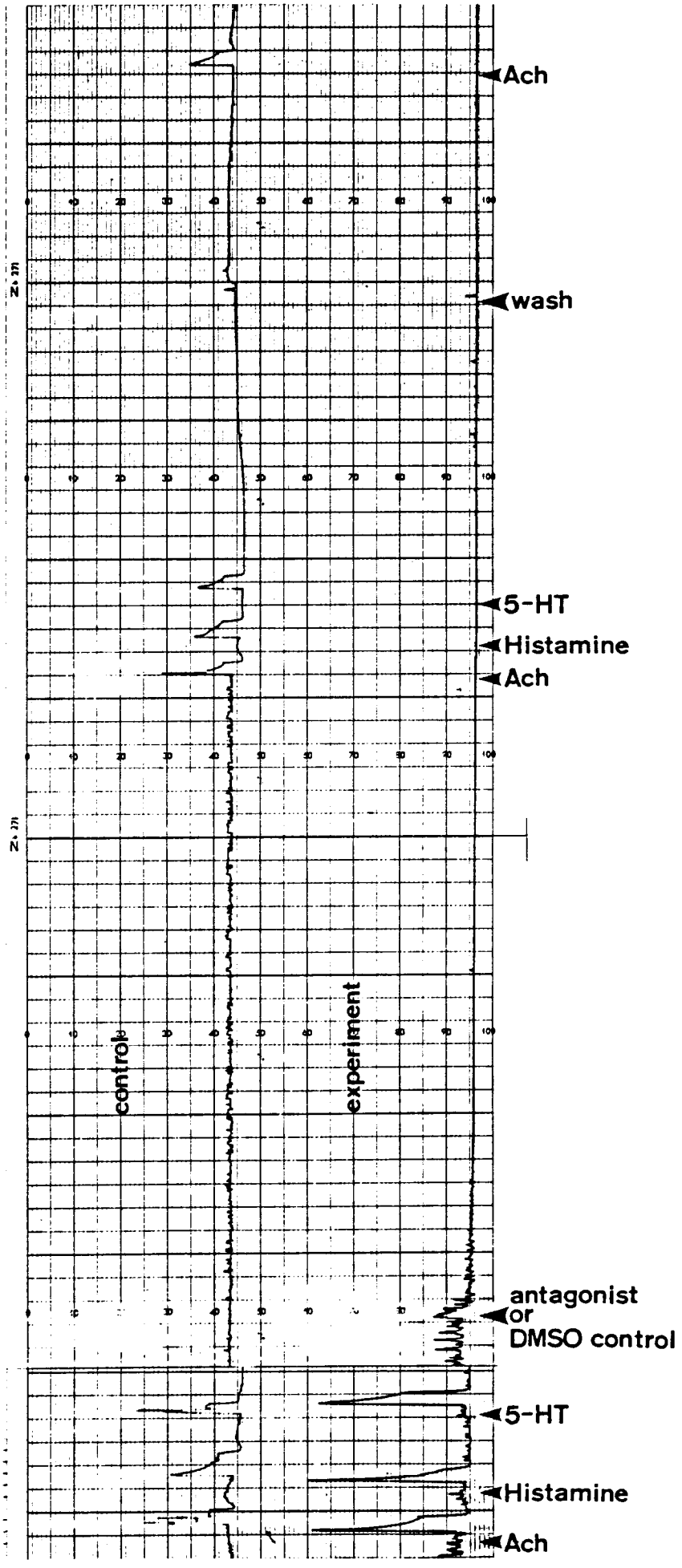
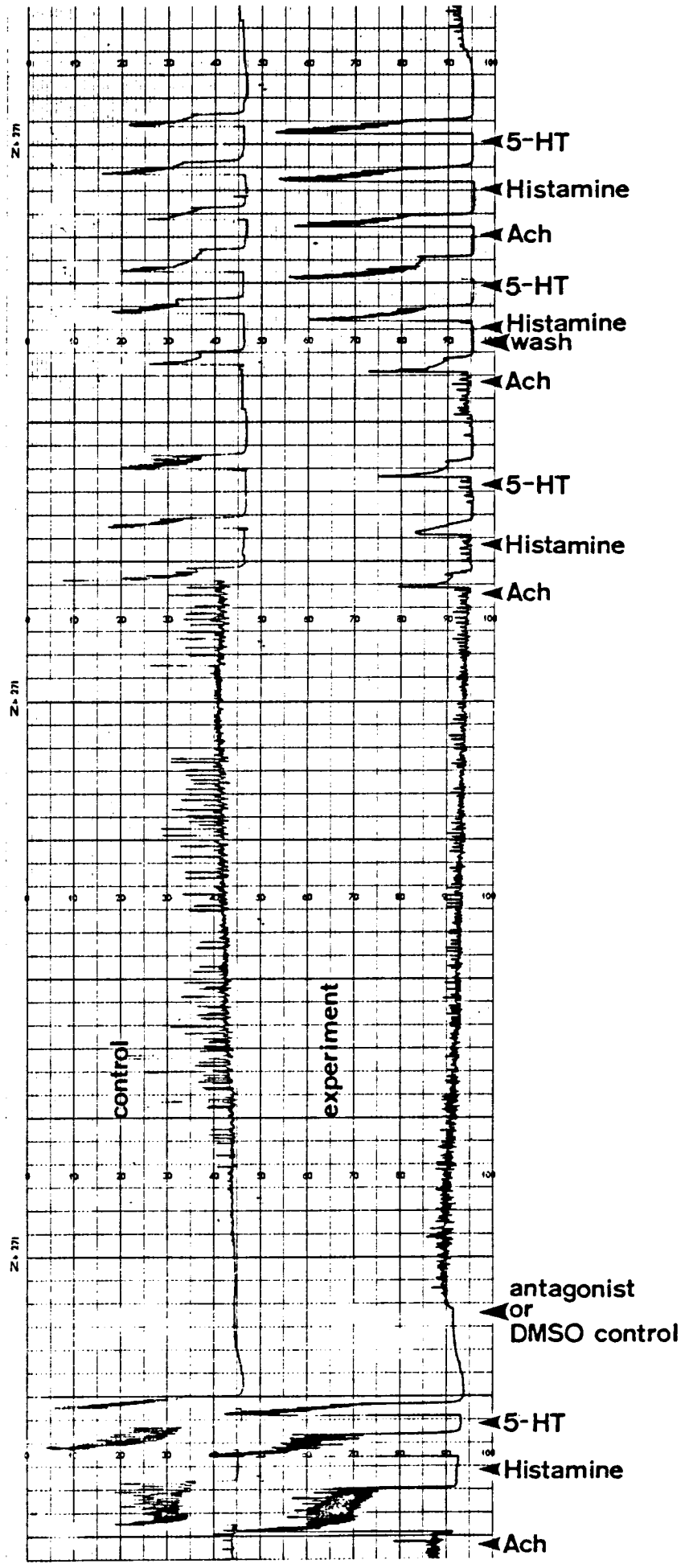


Figure 20

Spasmolytic activity of sub-fractions A30-34 tested at 10^{-4} g/ml (see Part III, 3)



2 EXTRACTION PROCEDURE

Dried leaves of Chrysanthemum parthenium were exhaustively extracted with solvents of increasing polarity: light petroleum, chloroform, methanol and water. Each of these extracts was tested for spasmolytic activity.

The methanol and water extracts showed no antagonism but the petroleum and chloroform extracts did show antagonism of acetylcholine, 5-hydroxytryptamine and histamine. In fact 100% antagonism of the agonists was obtained using a concentration of 10^{-4} g/ml of the latter extracts. These two extracts were therefore fractionated by column chromatography, and the fractions so obtained tested for activity.

Since a great number of these fractions showed antagonism of the agonists it was decided to proceed further only with the fractions showing 90-100% activity at 10^{-4} g/ml. These fractions were from the light petroleum extract and were further sub-fractionated and tested. Those sub-fractions still showing 100% antagonism were purified by preparative TLC or column chromatography as appropriate and where possible the active constituent isolated. In the majority of cases the sub-fractions contained only one major compound. The light petroleum extract possessed the greater activity so this extract was investigated first. These results are summarised in Figures 21 and 22.

From Figure 22 it can be seen that the greatest activity resided in fractions 51-61, 75-80 (C), 119-123 (E), 124-143 (B), 144-176 (A) and 177-200 (D). On examination of the TLC profiles of these combined fractions it was observed that each contained at least one component which gave a pink to purple colour after spraying with sulphuric acid

Figure 21

Extraction and isolation procedure of the light petroleum extract of Chrysanthemum parthenium

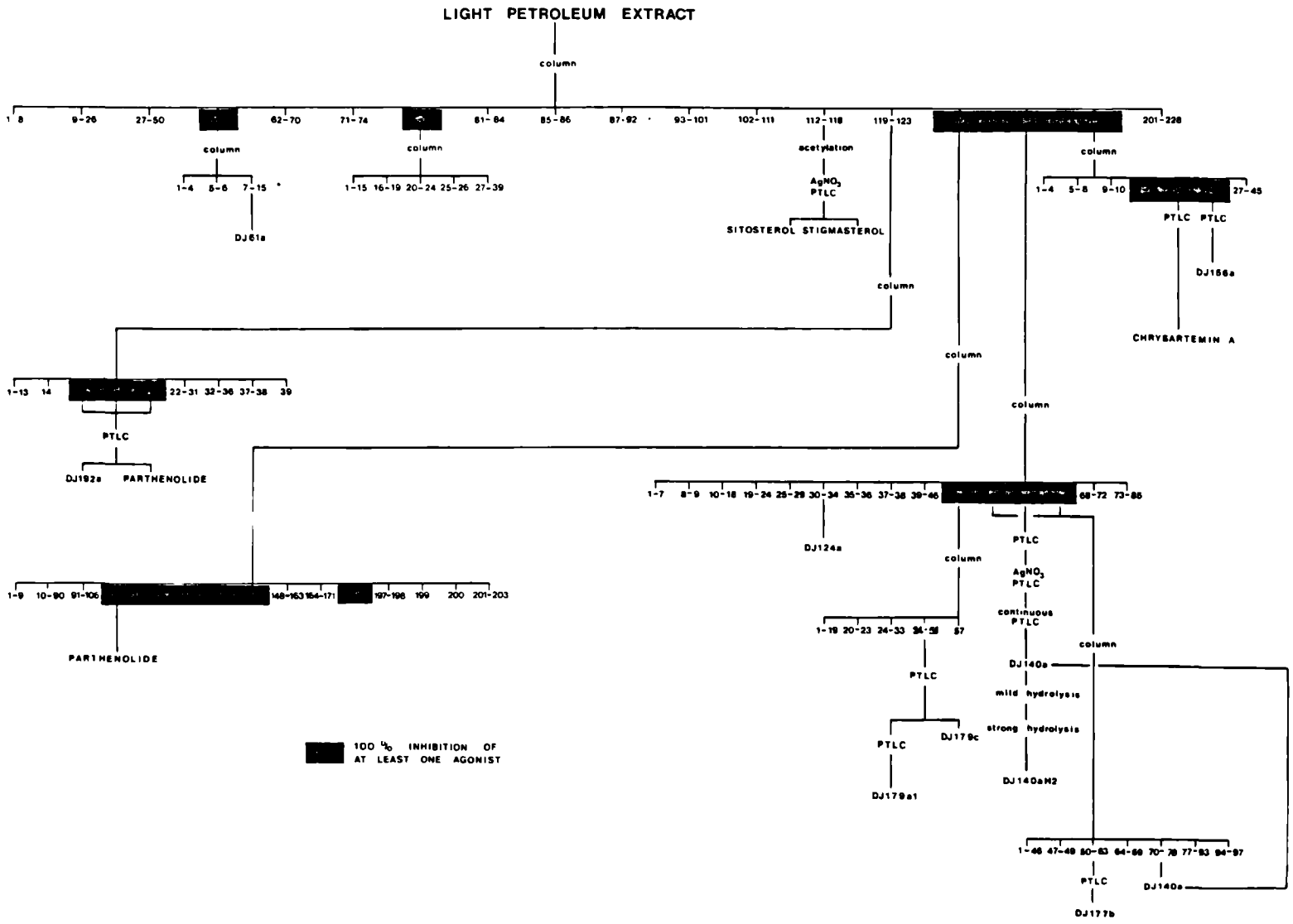
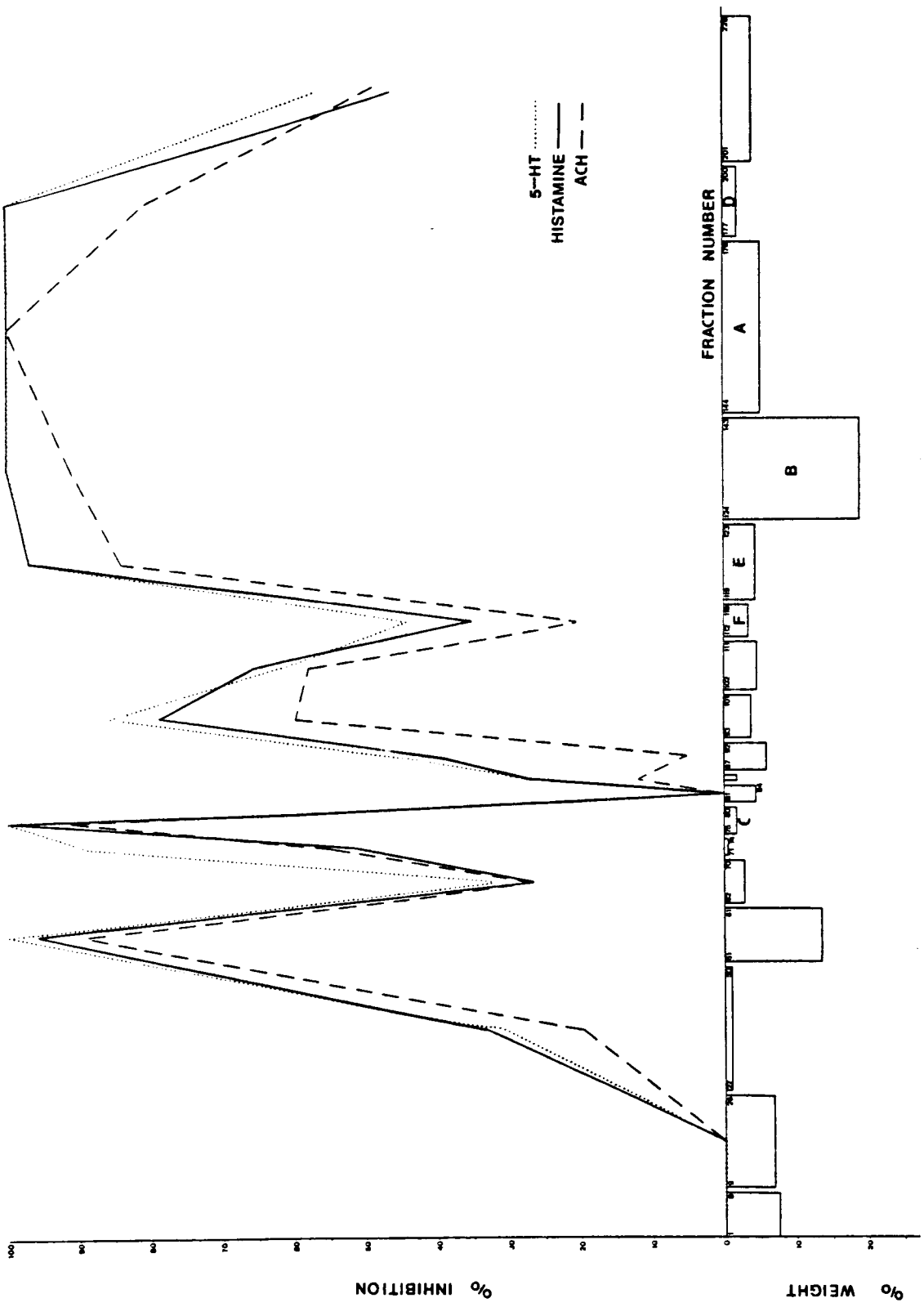


Figure 22

Spasmolytic activity and percentage weight of the fractions obtained from the column chromatography of the light petroleum extract of Chrysanthemum parthenium



followed by heating. Since such a reaction is often produced by terpenes and moreover, an authentic specimen of parthenolide obtained from Professor Sorm, gave a similar purple colour, it was considered that these compounds were likely to be sesquiterpene lactones. In addition from our knowledge of secondary plant metabolites found in the family Compositae the sesquiterpene lactones are likely to be the most biologically active group of substances.

In selecting the priority of the order of the work the weights of the fractions, their complexity on TLC examination, as well as their degree of inhibition of the three agonists were most important. Nevertheless, in fractions where isolation of a particular component, usually giving a pink to purple colour on spraying with sulphuric acid, appeared particularly simple, purification was carried out. This was done because even if the compound itself was found to be inactive valuable information could possibly be gained as to the nature of the hydrocarbon skeletons of the active substances. The active compounds all proved to be sesquiterpene lactones however and the non-active ones steroids, triterpenes or esters of long-chain fatty acids and not, as hoped, sesquiterpene hydrocarbons.

The three most active fractions were 124-143 (B), 144-176 (A) and 177-200 (D). Fractions 124-143 (B) represented by far the largest weight of the petroleum extract at 19% but by comparison with the sample provided by Sorm the major component corresponded on TLC with parthenolide. In addition, it showed only 91% inhibition of acetylcholine, but 100% activity against histamine and 5-hydroxytryptamine, these latter two agonists probably being more important in the pathogenesis of migraine. Similarly fractions 177-200 (D) showed less activity against acetylcholine and moreover represented only 2% in weight of the original extract.

Fractions 144-176 (A) were therefore investigated first, 100% inhibition being shown to all three agonists. In addition, at 5% of the original extract weight, it was hoped enough active material could be isolated to allow structure elucidation of the compounds. However, since these compounds were to prove to be the most complex of those isolated they will be discussed later. (Part II, 6)

3 KNOWN COMPOUNDS

Five known sesquiterpene lactones have been reported to be present in Chrysanthemum parthenium: parthenolide, chrysartemin A, chrysartemin B, santamarine and reynosin. In the course of the present study only the first two have been detected and isolated. This is because the latter three compounds are probably located in the chloroform extract of the plant which was not investigated here. It would be expected that the latter three compounds would be responsible for some of the activity shown by the fractionated chloroform extract. Furthermore, as already stated the present work was governed by biological activity, only those fractions having 100% activity to more than one agonist being examined in detail. The chloroform fractions showed less than 100% activity at the concentration tested so it may be concluded that the compounds present were less active, or present in lower concentrations than those in the light petroleum extract.

The possibility of the existence of more than chemotype cannot be discounted at this stage but the time required to investigate this suggestion was not available in the present study.

A PARTHENOLIDE

Parthenolide was first isolated from C. parthenium in 1959.[38]

It is the major secondary metabolite in the plant and is likely to be formed biosynthetically by epoxidisation of the 4-double bond of costunolide, the simplest sesquiterpene lactone.[44] Bearing in mind the large quantity of parthenolide found in feverfew it is reasonable to suggest that all subsequent sesquiterpene lactones are derivatives of parthenolide.

Govindachari et al. revised the structure of parthenolide in 1965[42] and the absolute configuration was proved by Quick and Rogers in 1976.[45]

In the present work the parthenolide-containing fractions 124-143 (B, in Figure 20) from chromatography of the petroleum extract were further chromatographed on a column of silica gel (see Figure 21). Parthenolide was located in sub-fractions B107-111 and B112-115 and as suspected these fractions corresponded with the major antagonist activity. It is interesting to note however that sub-fractions B164-171 and B172-196 appeared to show selective antihistamine activity and would therefore warrant further investigation.

Parthenolide crystallised from chloroform and hexane as colourless plates and was identical in all respects to an authentic sample provided by Sorm. Since the original structural work on this compound, the technique of ^{13}C NMR spectroscopy has become routinely available and has been found to be an extremely valuable tool in structural elucidation.[59b] Accordingly the ^{13}C NMR spectrum of parthenolide was obtained in the hope that its assignment would be of value in the structural elucidation of the new compounds to be described below.

The decoupled ^{13}C NMR spectrum of parthenolide in $CDCl_3$ solution

showed only fourteen clear signals. The position of the C-12 carbonyl signal could not be unequivocally located. It is not unusual that carbonyl signals are often very weak and not easily detected.[59c] The remaining signals were assigned with reference to the fully coupled spectrum, use of tables of chemical shifts[59d] and comparison with the data of known sesquiterpene lactones.[81,114-117] Unequivocal assignments were not possible in every case but the most likely are shown in Table 5.

B CHRYSARTEMIN A

Chrysartemin A[50,51] was isolated from fractions 177-200 (D) from the original light petroleum extract. These fractions showed 100% activity against 5-hydroxytryptamine and histamine. The combined fractions were further separated on a second silica gel column and most of the activity was shown to reside in the three sub-fractions D11-15, D16-20 and D21-26. The latter two sub-fractions showed 100% activity against all three agonists. Fractions D11-15 and D16-20 contained the same pink-reacting component but other different components were present in each set. In fractions D11-15 the pink-colouring component was not the major one whereas it was in fractions D16-20. In addition fractions D16-20 weighed 240 mg compared with 88 mg for fractions D11-15. Fractions D16-20 were therefore further purified by preparative TLC because of the greater likelihood of isolating the active principle. The major component crystallised from chloroform and hexane to give a compound the data of which was identical with those reported in the literature for chrysartemin A.[50,51]

Previously however the ^{1}H NMR spectrum had only been recorded in

Table 5

^{13}C NMR data for parthenolide (δ, $CDCl_3$)*

Chemical shift	Multiplicity in off-resonance spectrum	Carbon no.
16.97	q	14 or 15
17.24	q	15 or 14
24.11	t	8 or 3
30.64	t	3 or 8
36.35	t	9 or 2
41.19	t	2 or 9
47.65	d	7
61.45	d	5
66.36	s	4
82.38	d	6
121.08	d	1
125.24	t	13
134.51	s	10
139.21	s	11

*The signal for the carbonyl carbon (C-12) was not observed

dimethyl sulphoxide as solvent. With the increased sensitivity of Fourier transform instruments it was now possible to obtain a spectrum from a $CDCl_3$ solution and the data is reported in Part III, ^{8}14C(a).

4 STRUCTURAL ELUCIDATION OF DJ156a

The major component of sub-fractions D21-26 of fractions 177-200 (D) (see Figure 21) was a component which gave a pink colour after spraying with sulphuric acid and heating but had an R_f value different from that of chrysartemin A. This component also showed 100% inhibition of the three agonists. It was therefore isolated after purification by preparative TLC using chloroform:methanol (95:5) as developing solvent. The material (DJ156a) crystallised from chloroform and hexane to give 16 mg of white needles, m.p. 154°C.

The mass spectrum of this material did not show a clear molecular ion but showed peaks at m/z 248 (6.0%), 246 (18.0%) and 244 (13.5%) together with their appropriate ^{13}C isotope peaks. At first therefore it was thought the material could be a mixture of homologues. The ^{1}H NMR spectrum however showed signals for only one compound and the relatively high, sharp melting point also indicated homogeneity. On more detailed examination of the mass spectrum it was considered that the peaks at m/z 248-244 could really be fragment ions since a cluster of low intensity ions was visible at m/z 262-266. These could have given rise to the previous ones by loss of a molecule of water.

Extra weight to this suggestion of ready dehydration was given by examination of the infrared spectrum which showed a strong absorption

band at 3490 cm^{-1} for the presence of at least one hydroxyl group. The 400 MHz ^1H NMR spectrum however did not show a signal for a methine hydrogen (-CH-OH) and therefore the hydroxyl group or groups was likely to be tertiary. Tertiary alcohols are readily dehydrated[65c] and consequently rarely give a molecular ion peak in the mass spectrum. It was likely then that the peaks at m/z 244-248 could have arisen by loss of water from those at m/z 262-266. In addition however peaks at m/z 228 (19.8%) and 230 (10.0%) were observed, these perhaps being due to loss of a second molecule of water. The peaks at m/z 266 and 262 were of too low intensity to be measured but accurate mass measurement at m/z 264 indicated a molecular formula of $C_{15}H_{20}O_4$. This, together with the pink TLC appearance of the material after spraying with sulphuric acid followed by heating, indicated that the material was likely to be a sesquiterpene lactone. Since parthenolide ($C_{15}H_{20}O_3$) is the major sesquiterpene lactone in Chrysanthemum parthenium it is likely that the new compound is an oxidised derivative thereof.

The infrared spectrum of this new compound showed absorption bands at 1750, 1660 and 1640 cm^{-1}, characteristic of an α-methylene-γ-lactone. The presence of a conjugated lactone was confirmed by the strong ultraviolet absorption at 208 nm (log ε 3.95). In addition the 400 MHz ^1H NMR spectrum showed two very sharp doublets at δ5.54 and 6.25 (J = 3.5 Hz) for the hydrogens of an exocyclic double bond as in parthenolide.[42] These were assigned to H-13a and b. Such a coupling in this class of compounds, i.e. sharp doublets, is due to an allylic interaction with H-7.[53,54] The smaller geminal coupling (J = 1 Hz) is not observed unless an 8α-oxygen substituent is also present[55] (see Part I, 4A(a)).

In the present spectrum since these occurred as very sharp doublets

it was concluded that C-8 carried no α-oxygen function. This assumes that the compound was lactonised at C-6 as in parthenolide.[42]

No other low field signals were observed in the ^{1}H NMR spectrum showing that no additional vinyl hydrogens were present in the molecule. The next lowest field signal in the spectrum was a clear doublet of doublets at δ4.24 (1H, J = 12.3, 9.5 Hz) readily assignable to H-6 in sesquiterpene lactones. A spin-decoupling experiment by irradiation at δ4.24 caused another doublet of doublets at δ2.39 (J = 12.3, 12.3 Hz) to collapse to a doublet and simplification of the complex multiplet at δ2.70 (1H) to reveal a clear long range coupling of 3.5 Hz. The hydrogens giving rise to these signals are therefore both coupled to H-6 (but not to each other) and thus may be readily assigned to H-5 and H-7 respectively.

Further, the signal at δ2.70 showed a coupling of 3.5 Hz i.e. equal to the coupling observed for H-13a and b. This is obviously due to the allylic coupling with H-7. The signal at δ2.70 was thus unequivocally assigned to H-7. As extra evidence, on irradiation of this signal the doublets at δ5.54 and 6.25 did indeed collapse to singlets, so confirming the signal at δ2.70 to be due to H-7. From the complexity of the signal at δ2.70 and the appearance of the exocyclic methylene signals, H-8 must carry two hydrogens. Irradiation at δ2.70 also caused considerable simplification of the multiplets at δ2.16 and 1.47 which were thus assigned to the C-8 hydrogens.

The doublet of doublets at δ2.39 (J = 12.3, 12.3 Hz) must thus be due to H-5. On irradiation at δ4.24 (H-6) the signal for H-5 collapsed to a doublet (J = 12.3 Hz), implying that the carbon adjacent to H-5 carries only one hydrogen. Irradiation at H-5 caused the H-6 signal (δ4.24) to collapse to a doublet (J = 9.5 Hz) and also great simpli-

fication of the signal at δ2.63. This latter signal was thus due to the hydrogen on a carbon atom adjacent to C-5. The chemical shift of H-5 (δ2.70) excludes the possibility of C-5 also carrying an oxygen function.

As is likely from biosynthetic considerations the ^{1}H NMR spectrum of this material showed signals for two methyl groups. These were sharp singlets integrating for three hydrogens each at δ1.25 and 1.35. Since they are sharp singlets they must be due to tertiary methyl groups. Furthermore the chemical shifts of these indicate they are attached to carbons carrying oxygen.

C-5 carries no oxygen (from the chemical shift of H-5) on a methyl group, the methyl groups being tertiary. The compound must therefore be cyclised at C-5.

There are two possible modes of cyclisation of germacranolides i.e. to eudesmanolides or guianolides (see Figure 5). A eudesmanolide skeleton may be excluded by consideration of the chemical shift of the methyls and the fact that their ^{1}H NMR signals were singlets. In addition an eremophilanolide structure, which could arise from a methyl migration of a eudesmanolide (see Figure 5) was not admissable either since C-5 was known to carry a hydrogen atom. This, together with the low chemical shift of H-6, strongly indicated a guianolide skeleton. As with the eremophilanolide, an ambrosanolide or helenanolide structure, resulting from a methyl migration in a guianolide, were also clearly inadmissable (see Figure 5).

Incidentally, these data also provide extra evidence that the new compound is a C-6 lactonised sesquiterpene rather than being lactonised at C-8. As has been previously mentioned, irradiation at H-7 clearly located the hydrogen on the adjacent carbon atom to be at

δ4.24, i.e. H-6 in a C-6 lactonised compound or H-8 in an equivalent C-8 lactone. Irradiation at this shift further located the adjacent hydrogen to be at δ2.39. This must correspond with H-5 in a C-6 lactonised compound or H-9 in a C-8 lactonised compound. From the shape of the signal it has already been concluded that the carbon carrying this hydrogen forms one end of a ring function. This is very unlikely to be C-9 (i.e. in a C-8 lactonised compound) because it is highly likely that this material is derived by cyclisation of parthenolide (20) which has a 1(10)-double bond.

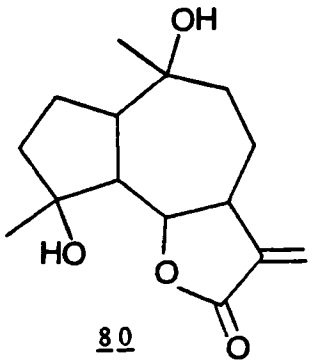
Since the compound was concluded to belong to the guianolide class the methyl groups must be placed at C-4 and C-10. The chemical shifts of the NMR signals of these methyl groups places oxygen atoms on these carbons. Furthermore, since it is known to be a guianolide derivative, the shifts indicate that these oxygen atoms must be present as hydroxyl groups rather than epoxides (the only functional groups admissable from the IR spectrum). Typically, shifts of methyl groups on carbons carrying tertiary hydroxyl groups are in the range δ1.11-1.40 while those on carbons bearing epoxides occur at δ1.48-1.62.[31i] This confirms the previous conclusions drawn from the infrared and mass spectra.

The signal at δ2.63 was assigned to H-1, C-4 having no hydrogens and the molecule being cyclised at C-5. As confirmation, irradiation at δ2.63 caused the doublet of doublets at δ2.39 (i.e. H-5) to collapse to a doublet (J = 12.3 Hz). Great simplification of the signal at δ1.62 (2H) also occurred on irradiation at H-1 (δ2.63) indicating that the former should be assigned to H-2.

The remaining signals in the ^{1}H NMR spectrum were those due to the hydroxyl hydrogens and those hydrogens at C-3 and C-9. These appear as complex signals at δ1.67-2.04 which cannot be individually

assigned.

The only structure compatible with these data is 80, 4α, 10β-dihydroxy-1β, 5α-gui-11(13)-en-12, 6α-olactone. The compound is given the trivial name partholide.



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This overall structure was confirmed by examination of the ^{13}C NMR spectrum. Fifteen signals were present in the fully decoupled spectrum. These were assigned with reference to the coupled INEPT ^{13}C spectrum, consideration of the chemical shifts and comparison with ^{13}C NMR data of parthenolide and other sesquiterpene lactones.[81,114-117] The data is given in Table 6.

Possible biosynthetic routes to the new compound are given in Figure 23.

Assuming the new material is derived from parthenolide, the stereochemistry at C-4, C-6 and C-7 is known. There are two asymmetric centres at C-1 and C-5 and therefore four possible isomers with regard to the ring junction. In addition there are two further possibilities since the stereochemistry at C-10 is not yet proved. Thus there are eight stereochemical possibilities which are summarised in Figure 24.

The ^{1}H NMR spectrum showed that the coupling constant between H-5 and

Table 6

^{13}C NMR data of DJ156a, 80 (δ, $CDCl_3$)

Chemical shift	Multiplicity in INEPT spectrum	J (Hz)	Carbon no.
23.51	q	125	14 or 15
24.25	q	120	15 or 14
25.01	t	112	2 or 8
25.33	t	150	8 or 2
39.34	t	130	9 or 3
43.77	t	128	3 or 9
47.22	d	140	7
49.78	d	130	1
55.35	d	130	5
74.72	s	-	10 or 4
79.88	s	-	4 or 10
82.74	d	150	6
120.37	t	163	13
138.45	s	-	11
169.37	s	-	12

Figure 23

Possible biosynthetic route to DJ156a

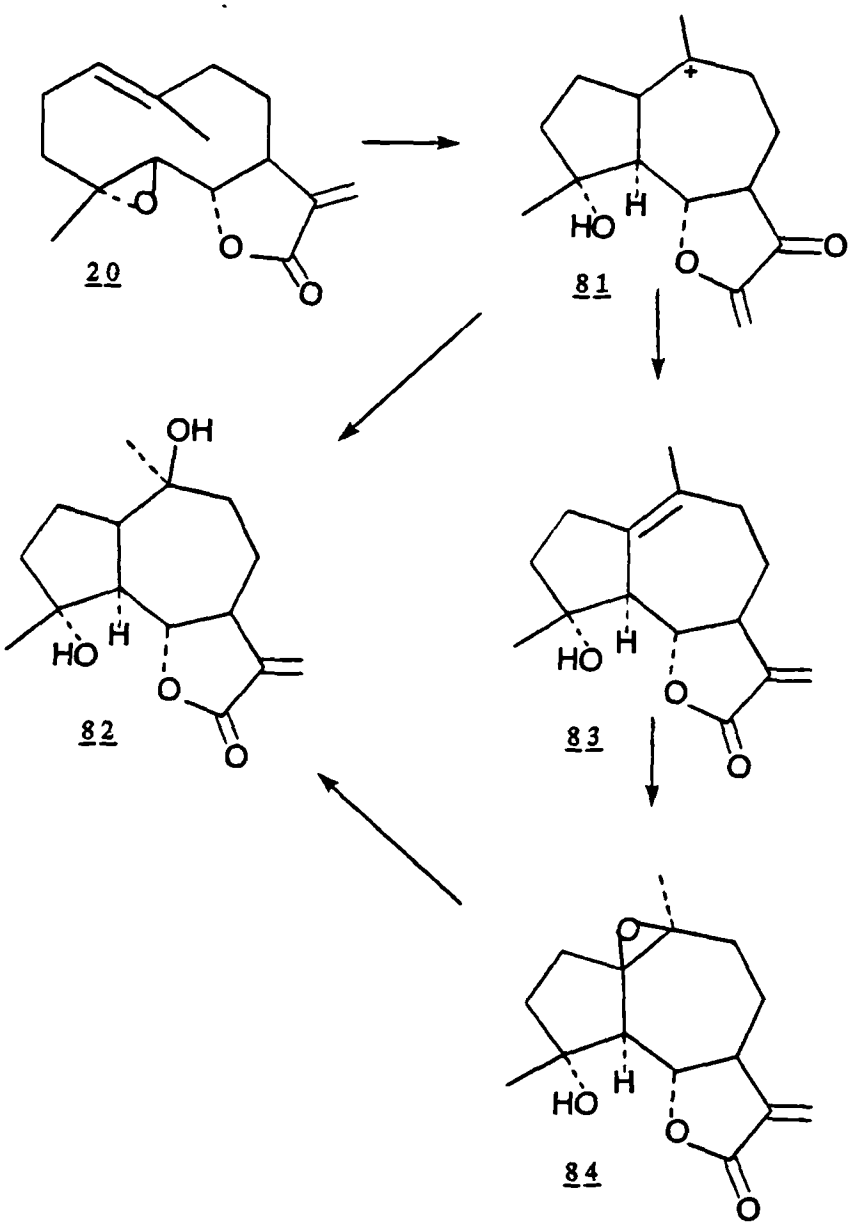
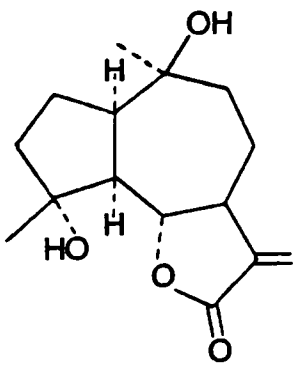
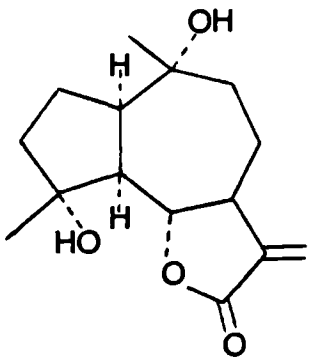
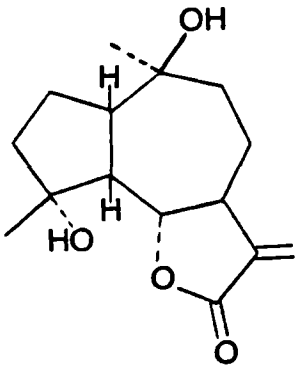


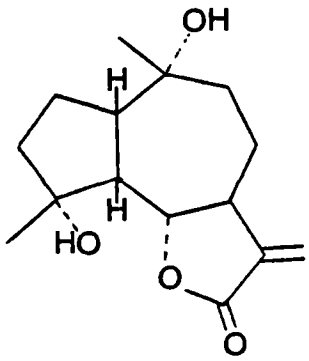
Figure 24

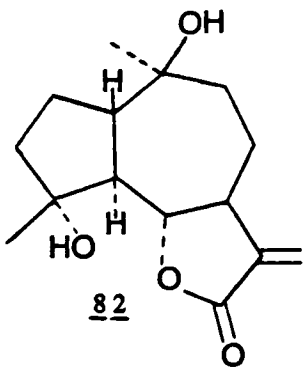
Stereochemical possibilities for the new guianolide DJ156a











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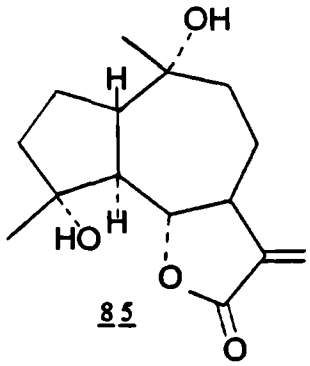

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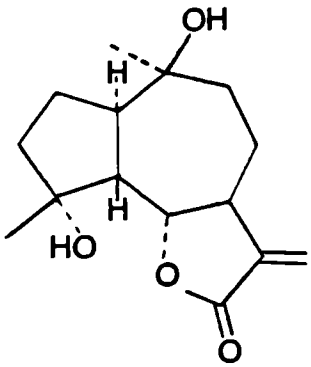
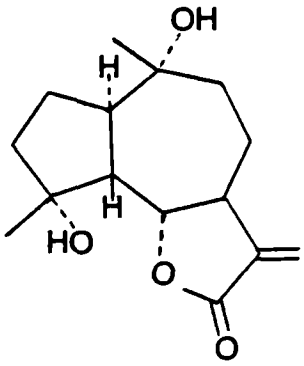




H-6 ($J_{5,6}$) is 12.3 Hz, $J_{1,5}$ is 12.3 Hz and $J_{6,7}$ is 9.5 Hz. From consideration of the Karplus relation,[65] such large coupling constants could only be produced by a structure with large dihedral angles between the appropriate vicinal hydrogens. Examination of Dreiding models showed that such angles could only be produced by a compound with a trans ring junction. Similarly, H-5 and H-6 must be trans. By analogy with parthenolide (the stereochemistry of which has been confirmed by X-ray crystallography[45]), since H-6 is β, the stereochemistry of H-5 is α and H-1, β. Moreover, that $J_{6,7}$ is 9.5 Hz also indicates the presence of a trans lactone which, since H-6 is β, fixes H-7 as α. This is of course expected if 80 is derived from parthenolide and has been assumed in the possibilities under consideration. This leaves only two allowable structures i.e. 82 and 85.

The final question to be resolved is thus the stereochemistry at C-10. This may best be postulated by a consideration of the likely mode of biosynthesis of the compound.

With reference to Figure 23, two routes are possible. The simplest involves the equivalent of acid catalysed opening of the 4,5-epoxide followed by trans-annular attack by the π-electrons of the 1(10)-double bond to form the guiane skeleton with a carbonium ion at C-10 (81). In solution, such a cyclisation would be expected to proceed in an anti fashion[118] and, since the C-O bond at C-5 is β, so produce a 5β ring junction. From the NMR data however there is no doubt that the hydrogen at C-5 is α so it may be concluded that the cyclisation proceeds by an enzyme-mediated process such that the germacranolide is adsorbed onto the surface of the appropriate enzyme which thus directs the steric course of the reaction to yield exclusively the 5α-product. This would involve attack of the π-electrons of the

1(10)-bond from the top of the molecule i.e. from the same side as the C(5)-O bond is broken. As the double bond is destroyed, rotation of the 1(10)-bond occurs to yield the product with a 1β hydrogen and the planar carbonium ion at C-10 as shown in 81. Finally, attack by OH^- would take place from the less hindered face of the molecule which is obviously also the top side so yielding 82 which has an α methyl group.

Similar considerations apply to the other possible route via the 9-olefin (83). Epoxidation to yield 84 would also take place from the less hindered (β) face, opening of which by a cis addition of hydrogen would also yield 82.

On examination of Dreiding models it is seen that the 7-membered ring may assume a conformation to minimise intramolecular interactions in which such an α methyl group is equatorial. This is thus also the thermodynamically favoured product. Incidentally, these deductions may be taken to strongly indicate that 82 is a true natural product since if it were an artefact produced in solution during extraction or purification, the material isomeric at C-5 could be expected to be formed.

5 STRUCTURAL ELUCIDATION OF DJ177b

Four components giving a pink to purple colour on spraying with sulphuric acid and heating were present in fractions 144-176 (A) from column chromatography of the light petroleum extract.

These fractions exhibited 100% activity to all three agonists and were therefore further separated on a second silica gel column and the sub-fractions tested for activity.

Sub-fractions A53-58 of the second column showed 100% inhibition of all three agonists and sub-fractions A66-67 100% inhibition of 5-hdroxytryptamine and histamine. Both sets of sub-fractions contained a different major pink-reacting component but several similar components. It was therefore decided the best way to separate the components, and to minimise losses, was to combine these two sub-fractions and rechromatograph them on a third silica gel column.

From this third column fractions Aa50-63 contained only one major component that appeared pink after spraying with sulphuric acid and heating. The second pink-reacting substance was isolated subsequently (fractions Aa70-76) and will be discussed later (part II, 6). The former fractions (Aa50-63) were further purified by preparative TLC and the major component crystallised from chloroform and hexane to give 8 mg of DJ177b as colourless needles, m.p. 116-117°C.

The mass spectrum appeared to show a molecular ion peak at m/z 264 (40.8%) with a ^{13}C isotope peak at m/z 265 (16.8%). However, small peaks were also observed at m/z 278 (0.2%), m/z 279 (2.6%) and m/z 280 (2.0%). Such a molecular weight together with the pink to purple colour produced on spraying with sulphuric acid and heating indicated a possible sesquiterpene lactone skeleton. This was confirmed by the presence of various characteristic spectral features described below.

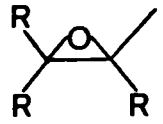
The infrared spectrum showed a strong absorption band at 3540 cm^{-1} indicating the presence of at least one hydroxyl group. In addition, as in compounds such as parthenolide[42] absorption bands were observed at 1760, 1665 and 1640 cm^{-1}, these being characteristic of an α-methylene-γ-lactone grouping. The presence of such a conjugated lactone was confirmed by the strong ultraviolet absorption at 215 nm (log ε, 4.10). Infrared absorption bands were also present at 1250,

900 and 805 cm^{-1} indicating the likely presence of an epoxide.[65b]

The 400 mHz ^1H NMR spectrum gave a total integral for eighteen hydrogens two of which gave broad signals at δ2.81 and 3.97 which could be due to hydroxyl groups. Unfortunately on the addition of D_2O precipitation of the compound resulted so confirmation by this method was not possible.

The ^1H NMR spectrum also showed two very sharp doublets (J = 3.5 Hz) integrating for one hydrogen each at δ5.45 and 6.18. These signals confirmed the presence of a conjugated exocyclic methylene group. Assuming, as in the discussion of DJ156a, that the material was a C-6 lactonised sesquiterpene, since these doublets occurred as very sharp signals, the possibility of an α-oxygen function at C-8 was excluded.[55]

Only one methyl group was present, the signal for this being a singlet at δ1.57. The methyl group is therefore tertiary. In the NMR spectra of sesquiterpene lactones such a chemical shift is indicative of the presence of a methyl group on a double bond or on a carbon atom carrying an oxygen, more particularly an epoxide.[31i] An accurate mass measurement at m/z 278 (corresponding with the molecular formula $C_{15}H_{18}O_5$) showed five oxygen atoms to be present of which two are accounted for in the lactone. The other three oxygen atoms must therefore be present as epoxides or hydroxyl groups. From the ^1H NMR spectrum there are likely to be two hydroxyl groups so the third oxygen was possibly present as an epoxide. This may be taken as extra evidence for the presence of a methyl located on a carbon atom carrying an epoxide. Furthermore, since no low field signals were observed for epoxide hydrogens as in chrysartemin A[50,51] (see Figure 25), it was assumed that the epoxide was fully substituted and a likely part structure was 86.



86

The chemical shift of the methyl group (δ1.57) indicated a guianolide structure as in chrysartemin A (C-10 methyl at δ1.58)[50,51] rather than a less rigid germacranolide as in parthenolide (δ1.32).[42]

Further examination of the NMR spectrum showed that a second exocyclic methylene group was present, the signals for which appeared as broad singlets integrating for one hydrogen each at δ4.88 and 5.21. (cf. reynosin, δ4.90, 5.03[49,50], dehydrocostuslactone, 87, δ5.08, 5.27[119] - see Figure 26). This accounts for the second methyl group usually present in sesquiterpenes which in this case has become oxidised to give a methylene function.

Figure 25

Structure of chrysartemin A showing the epoxide hydrogens and their chemical shifts in the ^1H NMR spectrum (δ, $CDCl_3$)

δ3.32, d, J = 1.3 Hz

δ3.57, d, J = 1.3 Hz

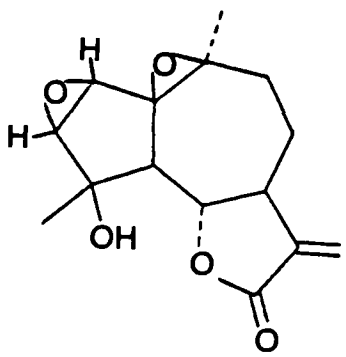
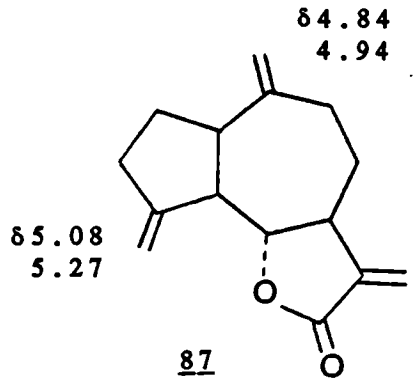


Figure 26

Structure of dehydrocostuslactone showing chemical shifts of the exocyclic methylene hydrogens



87

Biosynthetic considerations place methyl groups in sesquiterpenes at C-10 or C-4.[22-27] It was now necessary to decide on the class of sesquiterpene lactone to which the compound belonged.

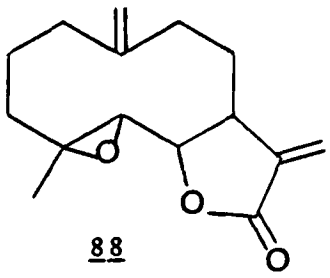
A eudesmanolide structure was not allowed. Only one germacranolide, 88, and two guianolide structures, 89 and 90, were possible (see Figure 27).

The similarity of the chemical shift data for the C-4 exocyclic methylene hydrogen atoms in the spectrum of dehydrocostuslactone (see Figure 26) with those of the present compound (δ4.88 and 5.21) was taken as evidence to place the exocyclic methylene group at position 4 (and not 10).

In dehydrocostuslactone the difference in chemical shift between the signals of the C-15 methylene group (at C-4) is 0.19 ppm whereas the corresponding figure for the C-14 methylene group (at C-10) is only 0.06 ppm. The large difference in the shifts of the 15-hydrogens is likely to be due to the proximity of the lactone ring (particularly the oxygen atom at C-6).

Figure 27

Possible part structures for DJ177b



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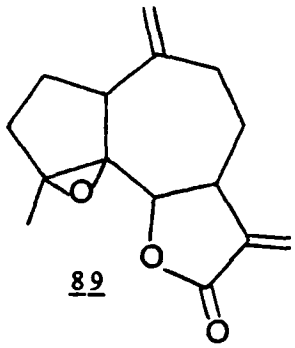

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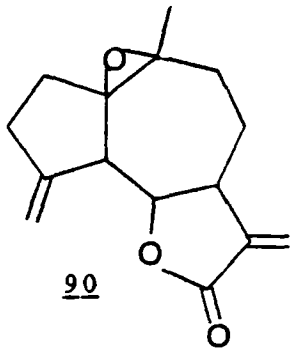

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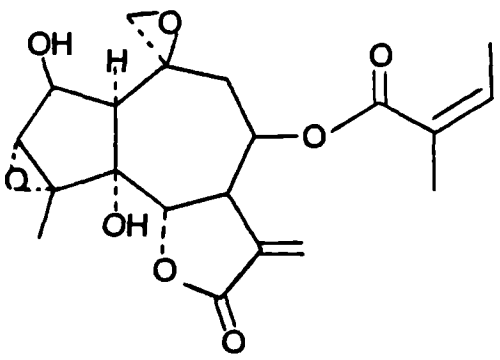
In the spectrum of DJ177b, the difference is even larger at 0.33 ppm which may not only be taken as evidence for the location of the methylene function at C-4 but also the presence nearby of additional functional groups causing greater non-equivalence of the hydrogen nuclei.

Spin-decoupling NMR experiments assisted in subsequent structural elucidation. Irradiation at δ4.88 caused the signal at δ5.21 to collapse and vice versa thereby showing them to be coupled. On irradiation at δ4.88 however and thus removing the signal at δ5.21, a doublet at δ5.23 (1H, J = 11.0 Hz) was more clearly seen. This was only partially observed in the fully coupled spectrum. At first sight this signal appeared to be due to the presence of a vinyl

hydrogen as in parthenolide[42] (measured in the present work at δ5.23, J = 12.5 Hz, long range coupling 3.0 Hz) and the signal at δ4.41 due to H-6. H-6 however occurs as a characteristically sharp doublet of doublets when there is a hydrogen at both C-5 and C-7 or as a clear doublet when C-5 carries no hydrogen atoms. The signal at δ4.41 in the spectrum of this compound was rather broad however. It was therefore concluded that the signal for H-6 was not in fact at δ4.41 but was more likely to be the unresolved doublet at δ5.23. This is a very low chemical shift for the C-6 hydrogen in the spectra of these compounds and thus indicated the presence of a grouping that was causing considerable deshielding.

For example, in eupachloroxin[120], 91, H-6 appears as a doublet at δ5.02, and in epi-eupatoroxin[120], 92, a doublet at δ5.08.

The epoxide cannot be present between C-3 and C-4 because of the absence of low field epoxide hydrogens but if the exocyclic methylene group was at C-4 with a hydroxyl group at C-5 the signal for the C-6



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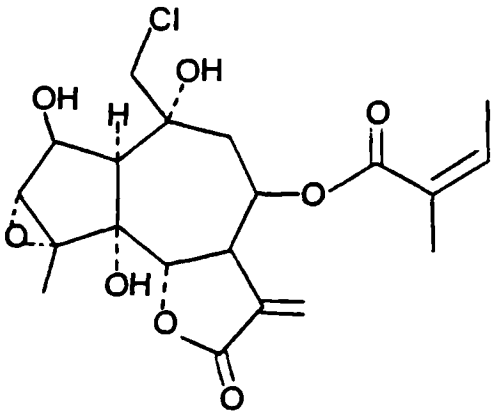

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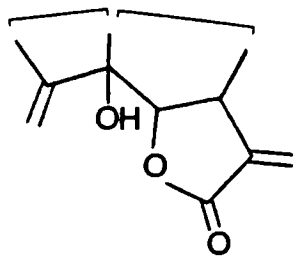
hydrogen could be deshielded to δ5.23 (see Figure 28). If this is the case, the germacranolide structure must be excluded since as the

signal for H-6 appears as a doublet there can be no hydrogens at C-5. C-7 bears the lactone and is never di-substituted and therefore must have one hydrogen.[31a]

Figure 28

Possible part structure for DJ177b



The placing of the exocyclic methylene group at C-4 is as earlier stated consistent with the chemical shift analogies with the spectrum of dehydrocostuslactone (Figure 26).

The coupling constant of 11.0 Hz for the signal at δ5.23 is consistent with a Dreiding model prediction for H-6 in the usual trans lactone. The signal at δ2.31 is a complex multiplet integrating for the hydrogen with coupling constants of 13.5 and 11.0 Hz. It was suspected therefore that this signal was due to H-7. Irradiation at this position caused a large reduction in the doublet at δ5.23. This, together with the coupling constant of 11.0 Hz proved the signal at δ5.23 to be due to H-6 and that at δ2.31 due to H-7.

Irradiation at δ2.31 also caused the low field doublets at δ5.45 and 6.18 (i.e. the C-13 hydrogens) to collapse to singlets. This was extra proof that the signal at δ2.31 was due to H-7, the coupling of the C-13 hydrogens being due to allylic coupling with the C-7

hydrogen.[53,54]

In addition, on irradiation at H-7, the signal at δ1.98 (integrating for two hydrogens) was substantially reduced to a broad doublet with a large coupling constant consistent with a geminal coupling. The signal at δ1.98 was thus assigned to the 8-hydrogens. Similarly, on irradiation at δ1.98 the signal at δ1.73 (integrating for two hydrogens) reduces to a broad doublet with a large coupling constant and was thus assigned to the C-9 hydrogens.

From the chemical shift of the signal at δ4.41 and its appearance it was assigned to the hydrogen on a carbon atom carrying a hydroxyl group. In pleniradin, the hydrogen at C-2 which also carries a hydroxyl group resonates at δ4.28.[31j] Similarly in epi-eupatoroxin it is at δ4.50,[31k,120] in eupatoroxin at δ4.30,[311] and in eupatundin at δ4.2-4.5.[31m] In zaluzanin A, which has a methylene group at C-4, the hydrogen at C-3 which also carries a hydroxyl group resonates at δ4.58.[31n] Thus a signal for a methine hydrogen on a carbon carrying a hydroxyl group at δ4.41 is in the normal range for guianolides.

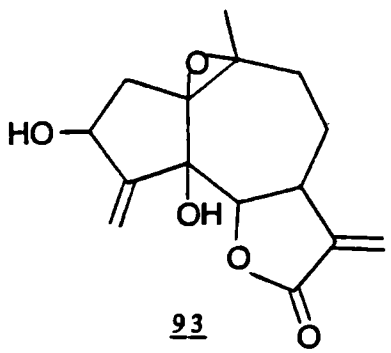
As C-8 bore no hydroxyl function it could only be placed at C-2 or C-3, C-5 having already been shown to have no hydrogen. Irradiation at the exocyclic methylene signal at δ4.88 caused the signal at δ4.41 to be simplified showing the corresponding hydrogen atoms to be coupled. A likely explanation for this is that the signal at δ4.41 due to a methine hydrogen was allylically coupled with the 15-hydrogens which, of course, could only occur if the secondary hydroxyl group and thus the methine hydrogen was present at C-3 (and not position 2). On this basis therefore the hydroxyl group was placed at C-3.

Confirmation was to some extent provided by the attempted acetylation of the material. Only a small amount of sample could be used for the

reaction and the ^1H NMR spectrum of the product showed signals for the starting material. Complete acetylation had therefore not occurred. Nevertheless a new set of signals was clearly visible including a sharp singlet at δ2.16 (assigned to the newly introduced CH_3CO_2-group) and a broad multiplet at δ4.60 identical in shape with the signal at δ4.41 in the spectrum of the pure alcohol. This was assigned to the methine hydrogen in the acetate which had been deshielded in the expected manner.[59e]

In the spectrum of DJ177b, irradiation at δ4.41 also caused a simplification in the resonance at δ2.18 (integrating for two hydrogens) and this latter signal was therefore assigned to the C-2 hydrogens.

The final problem was the location of the second hydroxyl group (which gave a signal at δ2.81) and the only position possible was at C-5. All the above NMR evidence led to the proposal of structure 93 for this compound.



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The molecular weight of this compound is 278 and indeed, as has been mentioned previously, the mass spectrum did show a small peak here as well as at m/z 279. As already stated accurate mass measurement at m/z 278 indicated the molecular formula $C_{15}H_{18}O_5$. The very small peak observed at m/z 278 is not unusual since the compound also

contains a tertiary hydroxyl group which would readily dehydrate.[65c] In fact, a much larger peak was observed at m/z 260 (8.0%), corresponding with ($M^{+\cdot}$ - 18).

The large peak at m/z 264 observed in the mass spectrum was concluded to have arisen from the loss of a methyl radical from m/z 279, i.e. (M+1).

A quantity of material sufficient to obtain a ^{13}C NMR spectrum to confirm the above proposal was unfortunately not available.

The problem of the stereochemistry of DJ177b is a difficult one. In the proposed biosynthetic route (see Figure 29) the existing stereochemistry is destroyed at both ring junction carbons. Furthermore, since a hydroxylase enzyme is likely to be involved in the introduction of the 3-hydroxyl group either epimer may result. Examination of the coupling constants in the ^{1}H NMR spectrum is of no value since C-5 bears no hydrogen atom to interact with H-6 (with fixed stereochemistry). Study of Dreiding models does not indicate which of the sixteen possible isomers of DJ177b is more or less favoured than the others so unfortunately no stereochemical proposals may be made other than to state that the C-6/C-7 junction is trans as in parthenolide.[42]

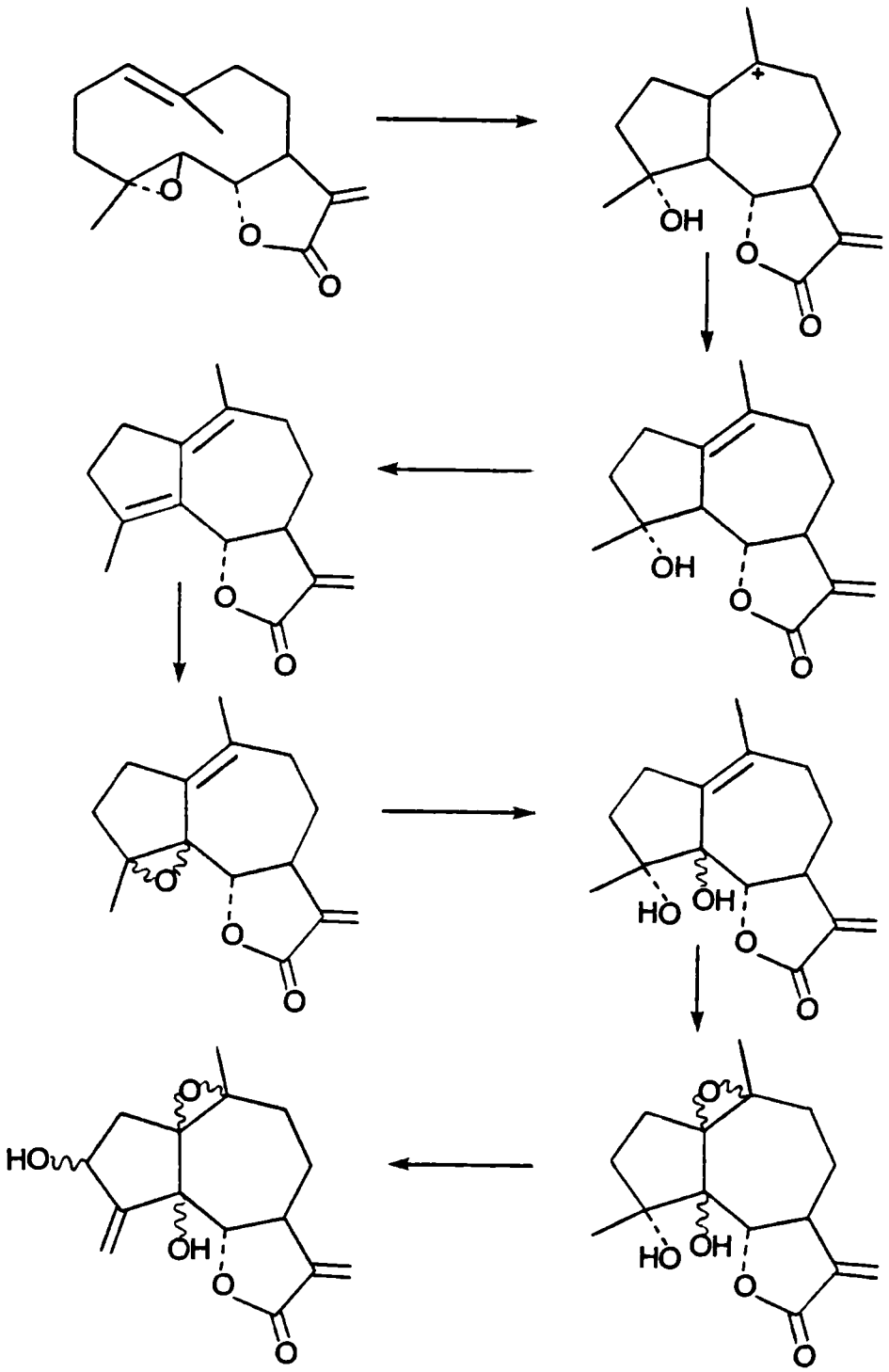
The compound, 9, 10-epoxy-3, 5-dihydroxyguia-4(15), 11(13)-dien-12, 6α-olactone is a new sesquiterpene lactone and is given the trivial name chrysanthemolide.

6 STRUCTURAL ELUCIDATION OF DJ140a

Four components giving a pink to purple colour on spraying with sulphuric acid and heating were present in the highly active light

Figure 29

Possible biosynthetic route to DJ177b



petroleum fractions 144-176 (A).

These fractions were separated on a silica gel column and the sub-fractions so obtained tested for activity. Sub-fractions A59-65, the major combined sub-fractions by weight, showed 100% inhibition of all three agonists. The thin-layer chromatogram of these fractions on spraying with sulphuric acid following by heating showed only one major purple component to be present. They were therefore separated by preparative thin-layer chromatography. The major isolated compound crystallised from chloroform and methanol to give colourless needles (26 mg) of DJ140a, m.p. of 211°C.

The infrared spectrum showed, as in the other compounds isolated, a very intense band at 1760 cm^{-1} characteristic of an α-methylene-γ-lactone with a weaker band at 1715 cm^{-1} which may be assigned to another carbonyl group, possibly an ester.

The presence of an α-methylene-γ-lactone was confirmed by the strong ultraviolet absorption at 210 nm (log ε 4.01).

The ^{1}H NMR spectrum showed signals with the general appearance of the other sesquiterpene lactones previously isolated. It seemed however that every group of signals was multiple, for example, the multiplet at ca δ4 (usually readily assignable to H-6 in C-6 lactonised γ-lactones) had many overlapping lines and apparently integrated for at least three hydrogens.

DJ140a thus appeared to be either a polymer with at least three sesquiterpene residues or despite its highly crystalline nature and high, sharp melting point, a mixture of closely related susbtances. Further information was provided by the ^{13}C NMR spectrum which, although difficult to count precisely, gave signals for about fifty carbon atoms. This showed at least three sesquiterpene residues

indeed to be present.

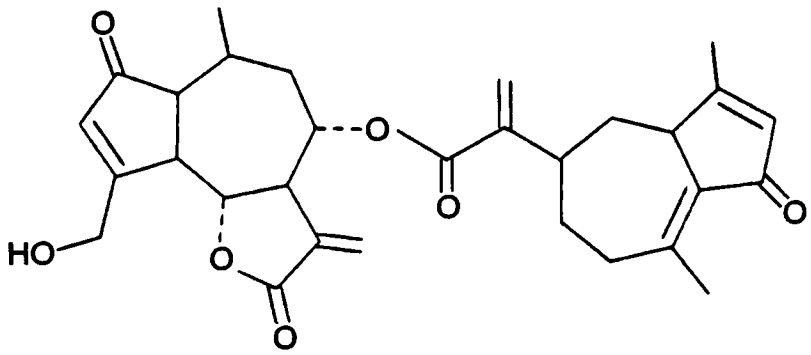
The mass spectral data on the other hand did not confirm this since, either with the use of various voltages of electron impact or using a chemical ionisation technique no corresponding molecular ion was visible. Thin-layer chromatography in many different solvent systems even using silver nitrate impregnated silica gel failed to show more than a single discrete spot.

It was thus considered that the material could be a mixture of conformational isomers. These are known to occur in the sesquiterpenes particularly those with a large and thus conformationally mobile ring such as the germacranolide isabelin (see Part I, 4A(d)).[60] It had been found before that recording the NMR spectrum at low temperatures using pre-cooled solvent to dissolve the crystals did allow signals for the predominate conformer of isabelin to be recorded.[60] Accordingly, a series of variable temperature ^{1}H NMR experiments was carried out on DJ140a but no significant change in the appearance of the signals could be detected.

Because of these results there was no option but to consider that the material was a molecule with more than one sesquiterpene residue which, for some reason, did not show a molecular ion on mass spectrometry. Such materials are unusual in nature but there is precedent for the existence of two types of such complex substances - novel carbocyclic compounds such as absinthin, 79,[121] and esters of a sesquiterpene acid and a sesquiterpene alcohol such as 1,10-dihydrolactucin-8-O-*iso*-hypoglabrate, 94.[122]

With the nature of these materials in mind, and since the infrared spectrum of DJ140a showed a band at 1715 cm^{-1} which may be due to an ester it was decided to try and hydrolyse the material in the hope of

simplifying the problem. If it were indeed an ester and so hydrolysable with base the spectra of the products should be easier to interpret. It is known however that on simple treatment with alcoholic alkali C-6 lactonised sesquiterpene lactones are destroyed and if an ester or hydroxyl function is present at C-8, relactonisation occurs at C-8 during the work up, as in Figure 30.[123]



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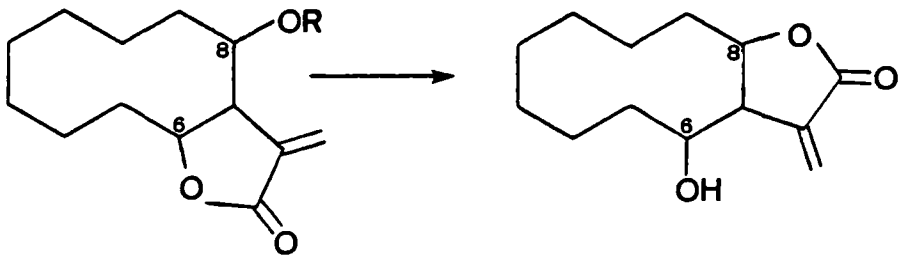
In an attempt to avoid this complication the material was first treated under very mild conditions (dilute aqueous potassium carbonate solution added to a dioxan solution of DJ140a).[123] No change occurred however even after heating for several days. The addition of further potassium carbonate solution caused no reaction and so a few drops of potassium hydroxide solution was added. Examination by thin-layer chromatography now did show the appearance of more polar products but before the starting material had been entirely consumed, the reaction was stopped and worked up in the usual way. Recovered DJ140a was recycled. Preparative thin-layer chromatography allowed isolation of a more polar non-acidic product, DJ140aH2, examination of the ^{1}H NMR spectrum of which was illuminating.

This compound was much simpler than the natural product and most signals were readily identifiable in terms of a dimeric sesquiterpene

derivative. Moreover, the fully coupled ^{13}C NMR spectrum showed signals for thirty carbon atoms.

Figure 30

Relactonisation of C-6 lactonised sesquiterpenes with a C-8 ester or hydroxyl in the presence of strong base



R=H, OCR'

A series of decoupling and INDOR experiments allowed unequivocal assignment of many signals. Some relevant chemical shift values are shown in Table 7, together with the appropriate values for the spectra of parthenolide, DJ156a and DJ177b.

From the data in Table 7 it is readily apparent that material DJ140aH2 comprises two different sesquiterpene moieties both possessing α-methylene-γ-lactones. In addition, one of the sesquiterpene positions is substituted with an 8α-hydroxyl group (column (b) in Table 7) because of the shift values (H-8 occurs at the relatively low field position of δ3.01) and the appearance of the signals for the C-13 hydrogens showing clear geminal coupling[55] (See Figure 31). The other lactone is not so substituted since its H-13 signals are almost identical with those of parthenolide and many other unsubstituted α-methylene-γ-lactones.

Decoupling experiments showed that H-8 (column (b) in Table 7) was coupled with signals at δ2.06 and 1.77 which are therefore assignable

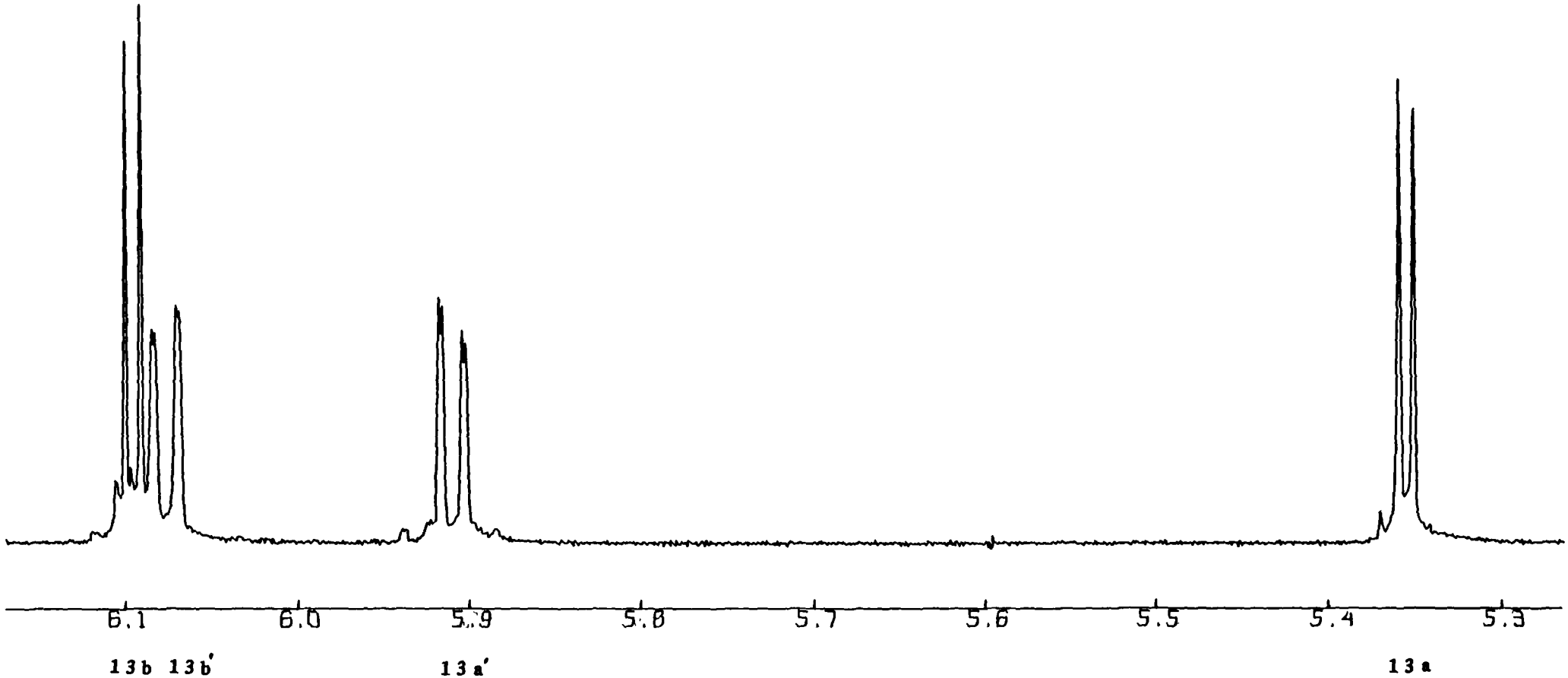
Table 7

Selected ^{1}H NMR signals of DJ140aH2, DJ156a, DJ177b and parthenolide (δ, $CDCl_3$)

Hydrogen number	DJ140aH2 a	DJ140aH2 b	Parthenolide	DJ156a	DJ177b
1	3.35	4.03	5.23	2.63	-
5	2.68	2.46	2.81	2.39	-
6	4.13	3.92	3.87	4.24	5.23
7	3.20	2.44	2.81	2.70	2.31
8	1.48,2.23	3.01	-	1.47,2.16	1.98,1.98
13a	5.35	5.89	5.64	5.54	5.45
13b	6.09	6.07	6.35	6.25	6.18
14	1.55	-	1.73	1.25	1.57
15	1.32	1.40	1.32	1.35	-
$C=CH_2$	4.91,5.15		-	-	4.88,5.21

Figure 31

Expanded ^{1}H NMR spectrum of DJ140aH2 showing clear geminal coupling of the C-13a' hydrogens but no such coupling of the C-13 hydrogens



to the two hydrogens at C-9. From the splitting of these H-9 signals it may be seen that they are not further coupled, i.e. C-10 carries no hydrogen atoms.

Only three methyl signals are visible in the ^{1}H NMR spectrum of DJ140aH2 (at δ1.32, 1.40 and 1.55 and thus are likely to be on carbons carrying oxygen) showing that one of the methyl groups in the precursor sesquiterpene lactone had been removed. A clear set of signals in the vinyl region however integrated for 2 hydrogens and may thus be assigned to another exocyclic methylene fraction, as in DJ177b, so accounting for the lost methyl group. It is not readily apparent at this stage however to which of the halves of the molecule this methylene group belongs.

The shifts of the H-6 signals at δ4.13 and 3.92 strongly indicate the presence of a germacrane skeleton since the corresponding signal in the guianes and eudesmanes normally appears at lower field (δ4.4-4.8 or even lower depending on substituents cf. DJ177b - 5.23).[31i] Furthermore, since in both cases the H-5 signals are sharp doublets and thus only coupled with the corresponding C-6 hydrogens, C-4 carries no hydrogen atoms in either case.

In summary then, at this stage, it may be stated that DJ140aH2 consists of a dimer compound of two non-identical sesquiterpene lactones of the germacrane class, one of which bears an 8α-hydroxyl group. Other substituents include an exocyclic methylene group, three tertiary methyls and various oxygen residues. C-4 is fully substituted in both cases and the linkage between the two residues is stable to base, i.e. it is not an ester.

It is appropriate to mention at this stage that the signal at δ3.01 in the (b) part of the molecule in DJ140aH2 was not present at this

shift in the ^{1}H NMR spectrum of the parent natural substance DJ140a. An identically shaped signal was however clearly visible at the much lower shift of δ5.19 characteristic of the methine hydrogen in esters,[123,124] so proving that DJ140a was indeed an ester of an acid and an 8α-hydroxy sesquiterpene. It would therefore be expected that such a material on hydrolysis would open and recyclise to the C-8 lactone as in Figure 30. There is no doubt however that the non-acidic hydrolysis product DJ140aH2 contains an 8α-hydroxy C-6 lactone. No explanation can be offered for this observation except that the period of hydrolysis allowed, despite involving potassium hydroxide, was short and the reaction stopped before completion to allow isolation and recycling of the unconsumed starting material. It may also be that, being a dimer, DJ140aH2 may be held in a stable conformation perhaps by hydrogen bonding from the other half of the molecule.

The linkage between the two halves is known not to be an ester and thus can only be an ether or acetal if oxygen is involved, or directly, via a carbon-carbon bond. For an acetal-type linkage an hydrated aldehyde would be involved which would necessitate oxidation of one of the methyl groups. This is clearly inadmissable since all four non-ring carbons are accounted for in the three tertiary methyl groups and one methylene function. Unless a novel carbocyclic skeleton is involved it is thus likely that the linkage between the two halves is via an ether.

From an examination of the ^{1}H NMR spectrum of DJ140aH2, all low field resonances have been accounted for except for two signals. These are a singlet at δ3.35 (1H) characteristic of methine hydrogens in epoxides (cf. δ3.32 in the spectrum of chrysartemin A - Figure 25) and a broad multiplet at δ4.03, for a methine hydrogen of an ether or

secondary alcohol. The couplings of this latter signal are 9.5 Hz, 5.5 Hz and 1.5 Hz. Such complexity may only be explained by postulating that the small coupling (J = 1.5 Hz) is an allylic interaction with the non-lactonic methylene group (δ4.91, 5.15, J = 1.5 Hz) similar to that observed with H-13a and b and H-7.

This gives a part structure of $-CH(OR)-\overset{|}{C}=CH_2$ and locates the methine hydrogen on a carbon adjacent to C-4 or C-10 in one of the germacrane residues assuming no methyl migrations have occurred. Such a rearrangement is unlikely to have taken place in the non-cyclised compounds.

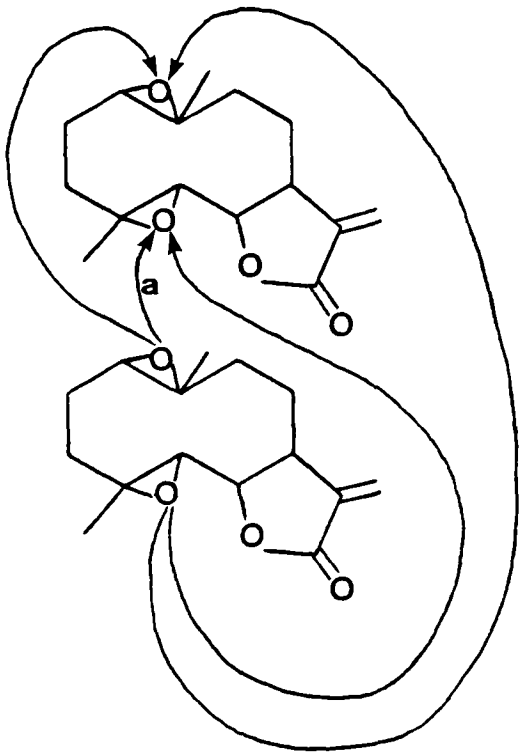
Because of the similarity of one set of the ^1H NMR signals to those of parthenolide and the co-occurrence of this material in C. parthenium it is not unreasonable to propose that DJ140aH2 is derived from parthenolide or a derivative. The newly formed linkage must not involve the lactone in either half since the appropriate ^1H NMR signals are characteristic. Since no vinyl signals are present it is likely that the corresponding epoxides are involved. Furthermore, the two halves are likely to be identical except that one bears an 8α-hydroxyl group.

Possible methods of the linkage formation are shown in Figure 32.

Attack by the 4(5)-epoxide of one molecule could take place either at the 1(10)- or 4(5)-epoxide of the other residue and similarly if the 1(10)-epoxide of the first was involved, this also could attack at two places (see Figure 32). Only if the 4(5)-epoxide reacts with the 1(10)-epoxide of the other (route (a) in Figure 32) is a dimer formed which contains one epoxide with a methine hydrogen that would resonate at δ3.35 in the ^1H NMR spectrum.

Figure 32

Possible methods of linkage formation in DJ140aH2



The initial product would thus be a carbonium ion at C-10 95 which may readily lose a hydrogen atom from the 10-methyl group to form an exocyclic methylene group as in 96, consistent with the part structure deduced from the ^{1}H NMR spectrum.

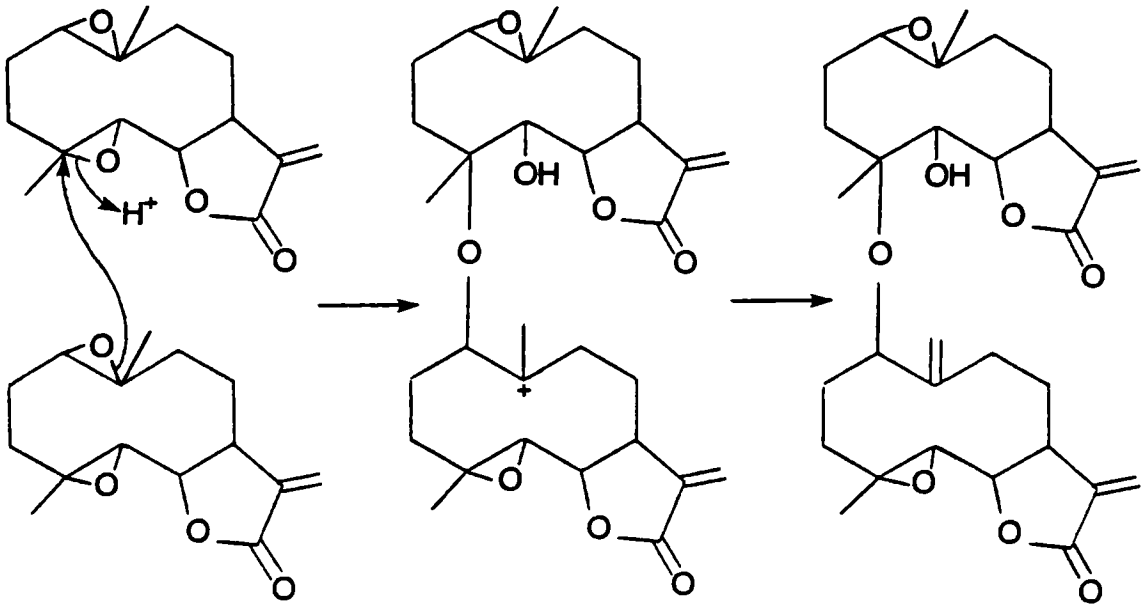
This also shows that it must be the epoxide -O-C(10) bond which breaks since if the -O-C(1) bond were cleaved no low field methine hydrogen would appear in the ^{1}H NMR spectrum. There are two possible ways in which the 4(5)-epoxide could open i.e. to give either 96 or 97 to which must be added (to one half only) an 8α-hydroxyl group.

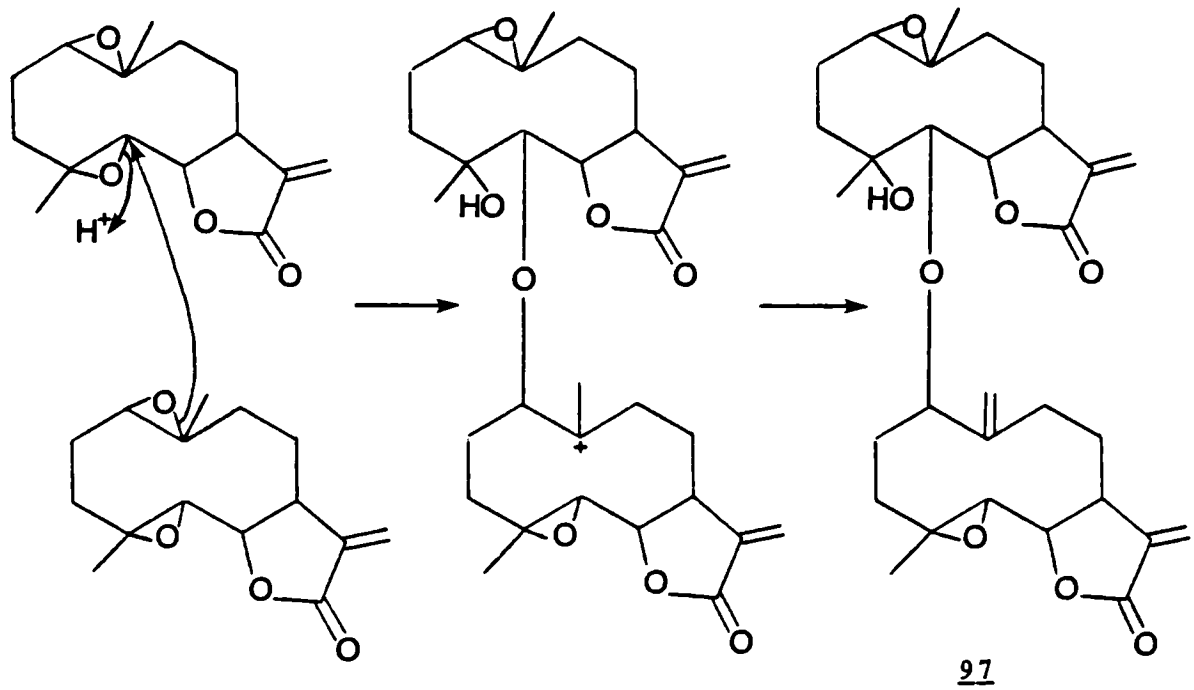
Ignoring for the moment stereochemical considerations there are thus four possible structures for DJ140aH2 - 98, 99, 100 and 101.

Figure 33 shows the proposed reaction mechanism resulting in DJ140aH2.

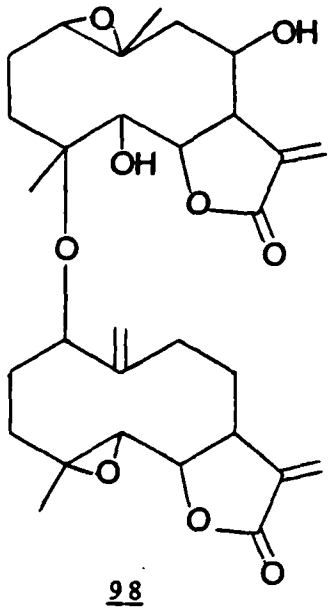
Figure 33

Proposed mechanism of the reaction resulting in DJ140aH2





These four structures differ only in the site of intermolecular linkage and the point of attachment of the 8α-hydroxyl group. 98 and



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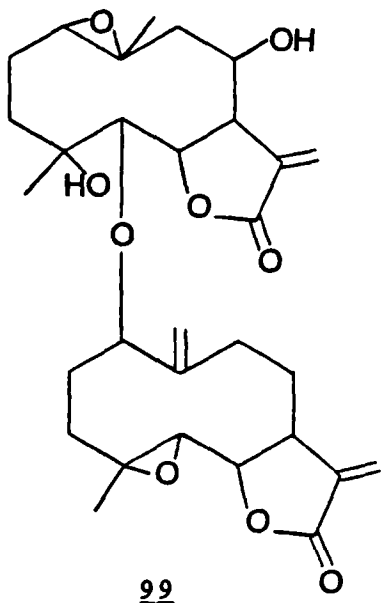

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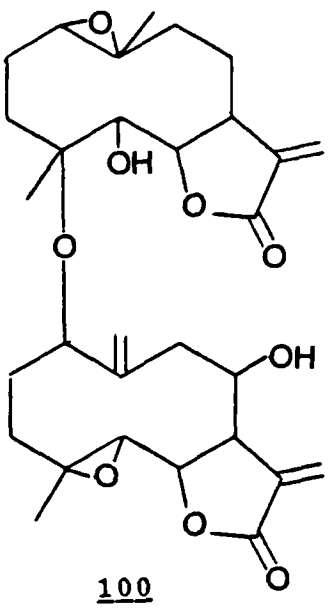

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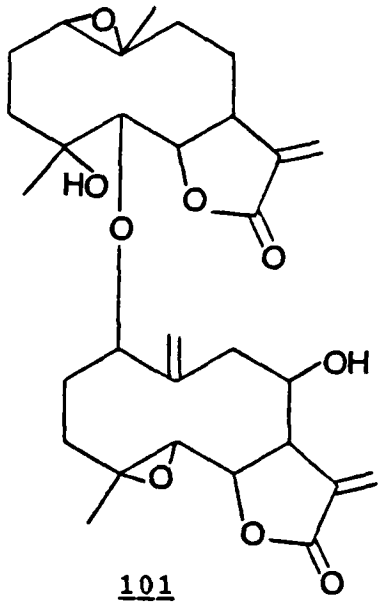


101

100 have a secondary hydroxyl group at 5 in addition to the 8α-hydroxyl. The ^{1}H NMR signals for H-5 occur at δ2.68 and 2.46 whereas H-8 in the portion which carries the 8-hydroxyl group resonates at the significantly lower field of δ3.01. This strongly indicates

either structure 99 or 101 where C-5 bears an ether or epoxide. The final problem is thus the placement of the 8α-hydroxyl function.

Although it is clear from the decoupling experiments to which set of H-5, H-6 and H-7 signals the comparatively low field of H-8β signal in the hydroxy-containing moiety belongs, the signals are not sufficiently distinctive to allow the two structures 99 and 10 to be distinguished. Useful information was however obtained from study of the mass spectrum.

Large peaks were visible at m/z 265 (7.1%) and 279 (12.9%) in the mass spectrum of DJ140aH2. If these arise as a result of simple C-O cleavage then the sum (544) should represent the mass of the parent molecule. Both structures 99 and 101 have formulae $C_{30}H_{40}O_9$ with molecular weight 544 so confirming the overall proposals. Furthermore, only if the lower half of the molecule carries the 8α-hydroxyl group, 101, can the two fragments at m/z 265 and 279 be produced. Accurate mass measurement at 265 and 279 indicated the molecular formulae of the two halves of the molecule to be $C_{15}H_{21}O_4$ and $C_{15}H_{19}O_5$ respectively, which confirms this proposal.

Further ions in the mass spectrum include peaks at m/z 281 (1.0%), 261 (1.3%), 247 (2.7%), 228 (5.0%), and 207 (8.9%). These may arise as shown in Figure 34.

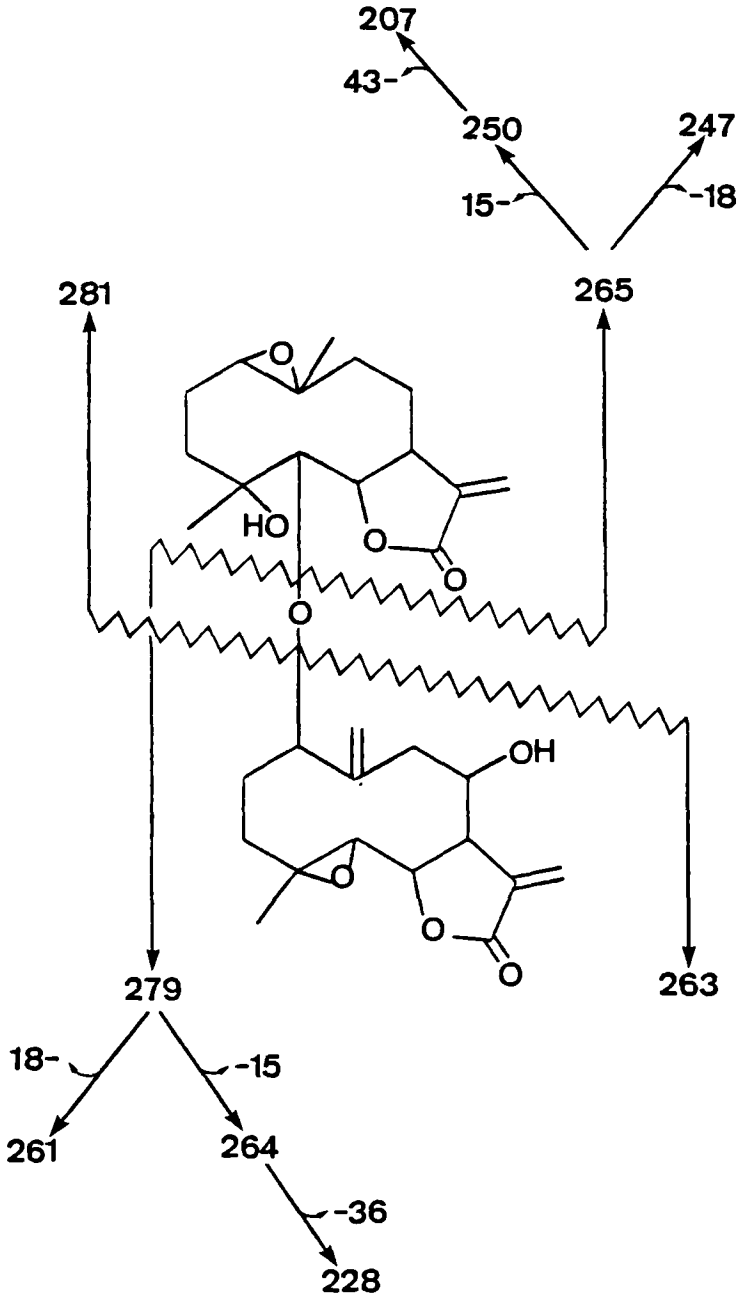
The ^{13}C NMR spectrum of DJ140aH2 was wholly consistent with the proposed structure 101.

With regard now to the stereochemistry of the molecule, from the ^{1}H NMR data, it is clear that H-5, H-6 and H-7 in both halves have a trans orientation to each other as in parthenolide. There is no reason to suppose that these centres are enantiomeric with those of

parthenolide so the most likely stereochemistry of the lactone moieties in both halves of the molecule is as in parthenolide. This also fixes the stereochemistry at C-4 and C-4'.

Figure 34

Mass spectral behaviour of DJ140aH2

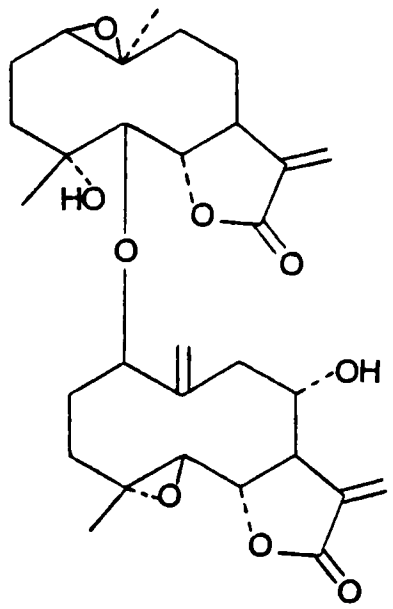


The only centres unaccounted for are C-1, C-10 and C-1'. From the proposed mechanism of the biosynthesis of the molecule, the C(1')-O-

bond will be orientated identically with the corresponding C(1)–O–epoxide bond and since the hypothetical epoxide is likely to be β (so that the C-10 methyl group may be equatorial) the most likely stereochemistry is shown in Figure 35. This material is thus 4'α, 5'β-epoxy-8'α-hydroxy-1'β-(1β, 10β-epoxy-4α-hydroxygermacr-11(13)-en-12, 6α-olactoyl-5β-yloxy)-germacra-10'(14'), 11'(13')-dien-12', 6'α-olactone.

Figure 35

Proposed stereochemistry of DJ140aH2



102

This is the first report, as far as the author is aware, of a sesquiterpene dimer of this type. Since acid (or the equivalent) has been implicated in its biosynthesis it is possible that the material is an artefact formed during work up or chromatography. This is considered highly unlikely since the monomer (parthenolide epoxide) has not been found in the plant.

It will be remembered that this material in fact was produced by

Figure 36

^{1}H NMR spectrum of DJ140a (δ, $CDCl_3$)

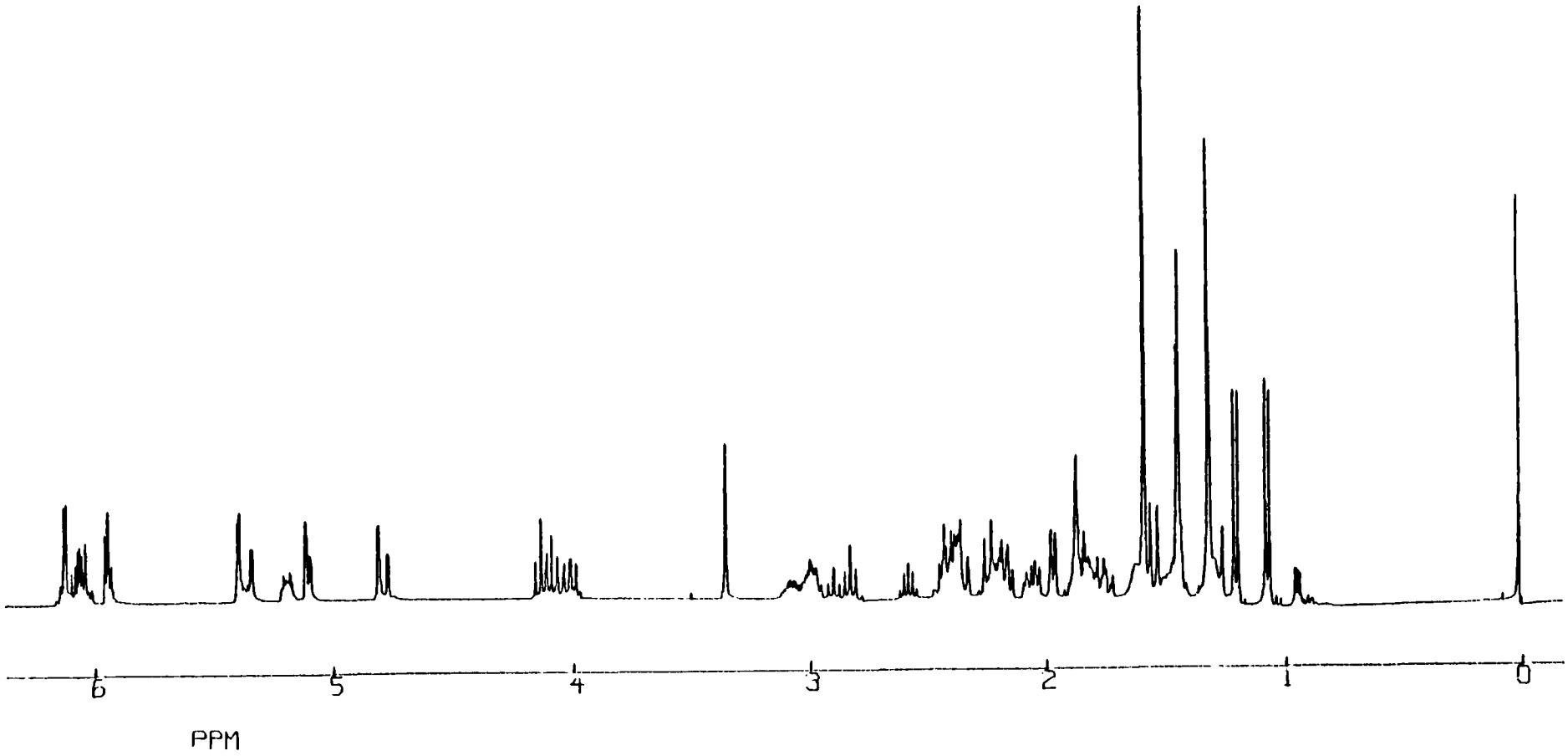
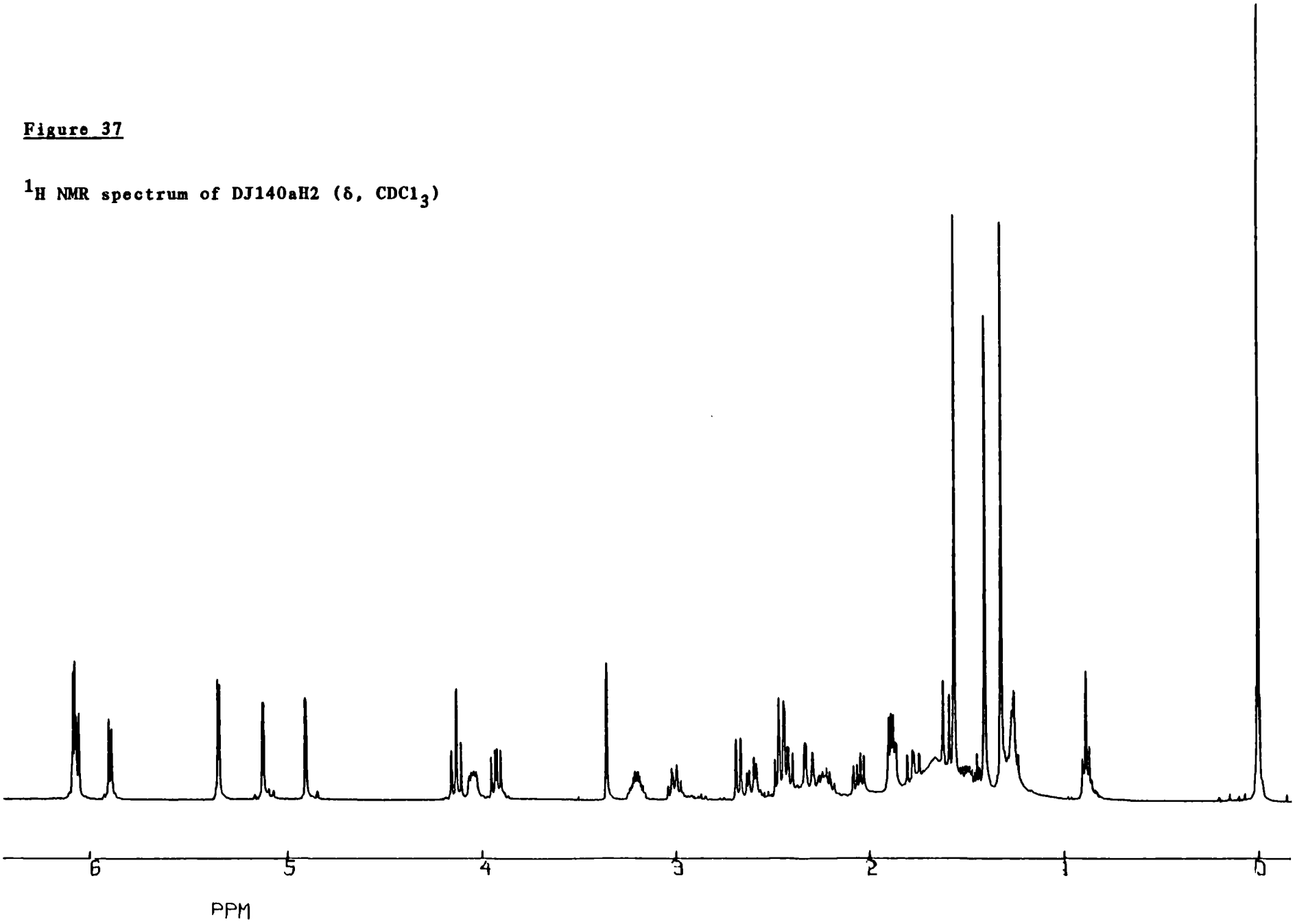


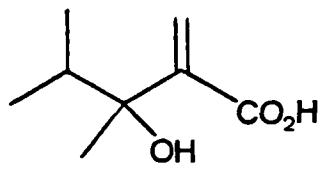
Figure 37

^{1}H NMR spectrum of DJ140aH2 (δ, $CDCl_3$)



basic hydrolysis of the highly active DJ140a. If the ^1H NMR spectra of the two substances are compared (Figures 36 and 37) it is apparent that a set of signals due to another sesquiterpene residue (together with other signals) is missing from the spectrum of DJ140aH2 compared with that of DJ140a (Table 9). Close inspection allows assignment of those signals in the spectrum of DJ140a and it is considered likely that they are caused by the presence of a molecule of a sesquiterpene acid derived by opening of the lactone of the lower half of DJ140aH2. This would be similar to 1,10-dihydrolactucin-8-O-iso-hypoglabrate from Hypothoeris oligocephala which is the ester between 1,10-dihydrolactucin and isohypoglabric acid.[122]

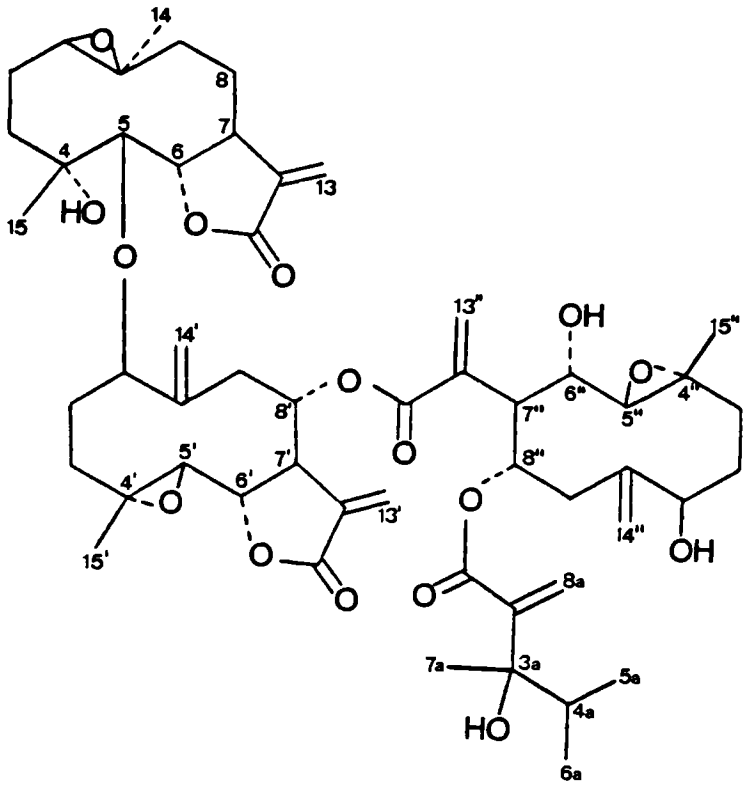
Of particular interest is a signal assignable to H-8 in the esterifying sesquiterpene acid which resonates at the low field position of δ5.36. This shows that the sesquiterpene acid is itself further esterified at C-8. Extra signals in the ^1H NMR spectrum of DJ140a are assignable to the fragments $(CH_3)_2$-CH-, -C(CH$_3$)-O- and CH_2=C(CO_2-)- from which it may be deduced that the structure of this second esterifying acid is 103, 3-hydroxy-3, 4-dimethyl-2-methylenepentanoic acid.



103

The proposed structure for DJ140a is thus 104, 8'α-[4'α, 5'β-epoxy-1'β-(1β, 10β-epoxy-4α-hydroxygermacr-11(13)-en-12, 6α-olactoyl-5β-yloxy)-germacra-10'(14'), 11'(13')-dien-12', 6α-olactoyl]-8''α-(3a-hydroxy-3a, 4a-dimethyl-2a-methylenepentanoyl)-4''α, 5''β-epoxy-1''β, 6''α-dihydroxygermacra-10''(14''), 11''(13'')-dien-12''oate and is

given the trivial name chrysanthemonin.



104

Selected ^{1}H NMR signals of the ester and alcohol are given in Table 8.

Confirmation of this structure was not possible by mass spectrometry since no peaks above m/z 381 were visible presumably due to ready decomposition of the molecule. A major ion at m/z 157 (8.6%) however may be assigned to the appropriate radical of the 8''α-ester group.

Such a complex sesquiterpene has never been reported in nature and in the absence of more chemical data this discussion cannot be considered a structural proof but the proposal is entirely consistent with the spectral evidence. Had more material been available it

Table 8

Selected ^1H NMR signals of DJ140a and DJ140aH2 (δ, $CDCl_3$)

Hydrogen number	DJ140a	DJ140aH2
1	3.36	3.35
5	2.18	2.68
6	4.13	4.13
7	3.08	3.20
8s	not assigned	1.48,2.23
13a	5.33	5.35
13b	6.07	6.09
14s	1.59	1.55
15s	1.32	1.32
1'	3.99	4.03
5'	2.42	2.46
6'	4.01	3.92
7'	2.82	2.44
8'	5.19	3.01
13'a	6.05	5.89
13b'	6.09	6.07
14's	4.78,5.09	4.91,5.15
15's	1.43	1.40
1''	2.99	
5''	2.38	
6''	4.09	
7''	2.89	
8''	5.36	
13''a	5.39	
13''b	6.13	
14''s	4.81,5.12	
15''s	1.44	
CH_3	1.07,1.21	
$(CH_3)_2-\underline{CH}-$	2.58	
$CH_3-\underset{\vert}{C}-O-$	1.33	
$-\underset{\vert}{C}=CH_2$	5.94	

would have been possible to isolate and attempt characterisation of the acidic hydrolysis products. This would also have enabled assignment of the ^{13}C NMR spectrum which was not possible in the present study because of the presence of many similar signals.

It is hoped that future studies will be carried out to confirm this novel structure, especially in view of the material's high non-competitive spasmolytic activity (see Part III, Table 19).

DJ140a was also isolated from sub-fractions Aa70-76 of the column chromatography of fractions A53-58 and A66-67 (see Figure 21). This material was combined with the DJ140a isolated previously.

7 COMMENTS ON DJ179a1 AND DJ179c

The remaining sub-fractions from the separation of fractions 144-176 (A) to show 100% activity against all three agonists were sub-fractions A47-52. These were further separated on a silica gel column and fractions Ab34-36 contained two major pink components on spraying with sulphuric acid and heating. Further separation by preparative thin layer chromatography yielded the two compounds DJ179a 1 and DJ179c (see Figure 21).

DJ179a1 is possibly a mixture of reynosin, chrysartemin A and a new sesquiterpene lactone, dihydroreynosin (which shows a methyl doublet in the ^{1}H NMR spectrum at δ1.40). There is also a possibility that the three compounds are joined as in DJ140a. DJ179a1 was isolated from the light petroleum extract in very low yield and a greater quantity of this material is probably present in the chloroform extract. Unfortunately the scarcity of material precluded further examination.

DJ179c was also a very complex sesquiterpene lactone containing an α-methylene-γ-lactone and, as with DJ179a1, could also not be studied in detail due to the very small amount isolated.

8 COMMENTS ON DJ124a

A mixture of fatty acid esters was isolated from sub-fractions A30-34 of the second column of fractions 144-176 (A) but was devoid of spasmolytic activity and so was not examined further.

9 THE REMAINING FRACTIONS TESTED FOR SPASMOLYTIC ACTIVITY

Fractions 75-80 (C) showed 100% inhibition of the agonists 5HT and histamine as well as PGE_2. The latter agonist was chosen because substances which inhibit prostaglandin synthesis are used in the treatment of migraine. After further separation on a second silica gel column however the activity appeared to have been lost. A similar situation was encountered with the remaining fractions from the petroleum extract to show 100% activity i.e. fractions 51-61. Only one major component was present in these and after further separation by column chormatography was isolated from sub-fractions 7-15. All the sub-fractions from this second column however were devoid of the significant activity shown by the original fractions. The compound isolated from sub-fractions 7-15 proved to be of a long chain fatty acid ester (DJ61a). Unfortunately, the increased purity of the compound resulted in its being barely soluble in dimethylsulphoxide. This solubility problem probably accounted for the loss of activity in this case as well as in fractions 75-80 (C).

This finding also made one aware of possible erroneous results

however. It seems unlikely that fats common in all plants could possess any significant activity even bearing in mind it being a possible precursor for prostaglandins.[125a] Since prostaglandins have not been isolated in any significant quantity[126] from plants to date it was concluded that the activity apparently shown by fractions 51-61 and fractions 75-80 (C) were false positive results.

The compounds, being fatty materials, were likely to have blocked the agonist receptor sites on the guinea pig ileum purely by forming a barrier. Since the membrane is fatty in nature the fractions under test would have had a greater affinity for the membrane than for the hydrophilic Krebs solution. On addition of the agonists after the 30 minute equilibrium period access to the receptor sites would be barred. Increasing the purity of the material however caused precipitation of the substance and so no film could be produced. No significant inhibition of the agonists was therefore recorded.

This problem showed some of the difficulties encountered with plant extracts of greatly varying polarity and perhaps over-simplified test procedure. Nevertheless it must be realised that the initial screening method was chosen to be very sensitive in order to pick up as much real activity as possible found in only small quantities.

Fractions 119-123 (E) showed good activity against 5-HT and histamine. These were further sub-fractionationed and tested for spasmolytic activity. After further separation by preparative TLC an unidentified triterpene and more parthenolide were isolated. It was concluded that the activity of these fractions was probably due to parthenolide.

The common plant sterols β-sitosterol and stigmasterol were isolated from fractions 112-118 (F). They did not show any significant

spasmolytic activity.

10 IN VITRO PHARMACOLOGICAL STUDIES ON THE EFFICACY OF CHRYSANTHEMUM PARTHENIUM IN MIGRAINE

It has been demonstrated in the present work that a light petroleum extract of C. parthenium showed very good spasmolytic activity in vitro with a possible indication of highest activity against 5-hydroxytryptamine. This result is of significance in the clinical application of the plant in migraine since 5-hydroxytryptamine is probably the most implicated of the agonists used in the pathogenesis of the disease.[108,110-112]

In addition the new compounds isolated showed inhibition of prostaglandin E_2.[125b]

11 PRELIMINARY TOXICITY STUDIES

Miss Julia Joce and Dr E S Johnson of Kings College, London have carried out limited feverfew feeding experiments with guinea pigs. 10 mg/kg to 135 mg/kg of freeze-dried feverfew mixed with normal feed (cf. human dose ca 1 mg/kg, based on a dose of three small leaves each weighing 25mg after drying per person per day) was fed to guinea pigs for periods of one to seven weeks. The ileum of each animal was then exised and tested as in the in vitro studies using acetylcholine, histamine, 5-hydroxytryptamine, nicotine, bradykinin and prostaglandin E_2 as agonists.

After feeding for one week the results showed a statistically significant non-competitive inhibition to 5-hydroxytryptamine but no effect on the response to bradykinin or nicotine was demonstrated.

The latter result implies that the plant was causing selective antagonism to 5-hydroxytryptamine at the receptor and not the nerve level.[127] Furthermore, no significant inhibition of acetylcholine, histamine or prostaglandin E_2 was obtained.

These preliminary results confirm the suspicion from the *in vitro* studies that the extracts and column fractions showed selective activity against 5-hydroxytryptamine.

Feeding for longer than one week caused no further effect to the response of the tissue. In addition, the tissues from the low-dose and high-dose animals gave similar responses.

No adverse effect on the tissues or behaviour of the test animals was noted even after administration of *ca* 135 times the equivalent human dose for a period of seven weeks.

12 CLINICAL STUDIES ON THE EFFICACY OF CHRYSANTHEMUM PARTHENIUM IN MIGRAINE

The difficulties of extrapolating results obtained with isolated tissue to clinical use in man are well known. This is particularly so in this case since the disease cannot be studied in live animals. The final test of efficacy of any drug must therefore rest in the clinic.

The code of medical ethics precluded prescribing feverfew to patients in the place of recognised treatments. In this case however, because of the widespread publicity the plant has received many migraine sufferers throughout Britain have been using feverfew. It was thus possible to study a group of volunteers who had already been using

the remedy of their own accord. This procedure did not infringe current legislation and permission was obtained from the Department of Health and Social Security to set up a limited clinical trial at the City of London Migraine Clinic, Charterhouse Square, London (DHSS number MF/8000/2025).

Only those patients who had been taking feverfew continuously for at least three months prior to the trial were eligible for enrolment. Ten patients of either sex between 18 and 60 years suffering from common, classical or mixed migraine were to be selected. They were also required to have a migraine history of at least two years with two to eight attacks a month before they started using feverfew. If any of the patients were taking other drugs such as alpha- or beta-blockers, tranquillisers, antidepressants, non-steroidal anti-inflammatory agents used prophylactically or 'antimigraine drugs' for example ergotamine, likely to interfere with the study, they were excluded. Patients who were pregnant or had known mental illness were also not allowed to participate.

The study was a double-blind, placebo controlled, crossover trial. The patients were randomly allocated either feverfew capsules containing their equivalent daily dose (on the basis that one 'normal sized' leaf weighed 25 mg when dried) or placeo capsules. After twelve weeks the patients crossed over to the other treatment. Venous blood (20 ml) was taken on enrolment and four weekly thereafter. The blood was analysed for liver and renal function and general haematology. The patients were required to record each migraine attack on diary cards.

Unfortunately, because of severe cramping experienced by one patient the code had to be broken when only four patients had been enrolled. This occurred in the fourth month when the patient was on placebo

capsules. There was no previous history of cramps and the possibility of withdrawal symptoms from the feverfew are to be investigated. A study of only four patients is obviously not statistically valid but it is hoped that a larger study will soon be carried out.

A much more valid clinical study is at present being undertaken by Dr E S Johnson of Kings College, University of London. At the time of writing, he has received 277 completed questionnaires from patients who are taking or have taken feverfew for migraine. A typical case history obtained from a questionnaire is outlined below.

A housewife now aged 75 began taking feverfew on the 27th of May 1978 after hearing of it from Mrs Jenkins of Cardiff. She takes one large fresh leaf or two small ones in a sandwich every day at 11 a.m. Consumption of the plant caused no change in bowel habit, appetite, sleep, mood, breathing, heart beat or weight.

She did not suffer from hot flushes, palpitations, mouth ulcers, indigestion, jaundice, bleeding gums or excessive thirst while taking feverfew.

The only side effects she attributed to feverfew were a skin rash on her face, increased urine output and swollen ankles. These appeared within a year of using the plant.

When she began taking feverfew her migraine headaches (diagnosed as classical by her doctor) became much less severe and she experienced less nausea with them. She has only taken Panadol for the headaches.

In April and May 1978, i.e. prior to taking feverfew, she suffered 8 migraine attacks. From the 27th May to the end of December 1978 i.e. 7 months, she suffered 12 attacks. In 1979 she had 18 attacks but in

1980 she stopped taking the plant because of the skin rash and suffered 32 attacks. In 1981 she began using the plant again and experienced only 10 attacks.

There seems to be little doubt that feverfew is having a beneficial effect in this case.

These questionnaires are being processed by computer and 60 patients are to be tested for general haematology, urine, liver and renal function.

13 RELATIONSHIP BETWEEN STRUCTURE AND ACTIVITY

It has recently been proposed by Collier et al.[128] and Makheja and Bailey[129] that Chrysanthemum parthenium acts in migraine by inhibiting prostaglandin synthesis. Makheja and Bailey allege that a phosphate buffer extract of feverfew inhibits platelet aggregation and hence the release of arachiodonic acid and its conversion to the prostaglandin thromboxane A_2. On repeating their experiment Dr E S Johnson could get no such inhibition even at a much higher concentration of plant extract.

All findings described in the present work point to the presence of sesquiterpene lactones in the plant as being responsible for the biological action. A phosphate buffer would not extract such organic compounds. In addition no significant inhibition of prostaglandin E_2 was found in the ilea of guinea pigs who had been eating the plant, this being more applicable to the human situation. The evidence is heavily weighted in favour of the sesquiterpene lactones as being responsible for the activity of feverfew.

The active compounds isolated in this study have certain structural

features in common. They all possess an α-methylene-γ-lactone moiety capable of undergoing a Michael-type addition reaction. (Part I, Figure 16). The presence of this grouping has been shown to be important in other activities shown by these compounds.[73,84,91,103]

The most active compound is DJ140a. This is a very large molecule with three C-11 methylene groups and three epoxides all capable of binding irreversibly to an enzyme in much the same way as has been proposed for the biosynthesis of the dimer DJ140aH2. Such an irreversible mechanism may account for the non-competitive antagonism observed for these compounds. Undoubtedly the presence of other active sites also enhances activity as has already been proved in the case of ketones, esters and double bonds in cytotoxity studies.[73]

These compounds are highly reactive and it seems likely that much semi-synthetic work is called for to produce a clinically effective substance. It is well known that α-methylene-γ-lactones cause contact dermatitis[130] and the most frequently encountered side effect in patients taking feverfew is mouth ulceration.

The presence of many similar sesquiterpene lactones in feverfew may act in a synergistic manner lowering the toxic effects. Conversely, administration of a pure compound could enhance such cytotoxic actions and attempts to reduce the side effects could result in loss of all activity.

14 CONCLUSION

The author has no doubt that Chrysanthemum parthenium is an effective prophylactic treatment for migraine. Discussions with patients indicate that it may prove to be also of as great or even greater

value in the treatment of rheumatoid arthritis.

The present study has demonstrated that the plant possesses marked spasmolytic activity which can be extrapolated to its clinical efficacy in migraine. The active constituents isolated thus far have all been sesquiterpene lactones. This class of metabolite, of relatively recent interest, is known to show a wide range of pharmacological activities. In the future they may rival the alkaloids as the most important group of plant-derived biologically active constituents.

The present work has resulted in the isolation of substances with novel structures. These would be of basic phytochemical interest even if they had no promising biological activity. They also provide a basis from which the synthetic chemist could work either by modification of the molecule to increase the therapeutic index, or, as a template for similar totally synthetic compounds. Structure activity relationship studies could result in a more thorough understanding of the aetiology of migraine.

Clearly this process is extremely costly both in time and money. Sadly, in these times of economic recession, the development of new drugs is losing the battle against the reformulation of existing drugs. Furthermore the unfounded scepticism of natural products ensures that projects dedicated to the study of plants with a long folk lore history are drastically under-supported. Many potential sources thus remain untapped.

It is the author's hope that the promising results of this study will provide encouragement for similar projects so that with a rational exploitation of the world's natural resources and an understanding of the real relevance of the study of folk-lore the outcome may be safe and effective treatment of disease.

PART III

E X P E R I M E N T A L

1 GENERAL DETAILS

A CHROMATOGRAPHY

(a) Adsorbents

Silica gel was the adsorbent used for all chromatography. The silica gel used for column chromatography was Kieselgel 60 (Merck) and for thin-layer chromatography (TLC) Kieselgel G (Merck) and Kieselgel GF_{254} (Merck). TLC plates for routine work, both analytical and preparative, were prepared by mixing 15 g Kieselgel G and 15 g Kieselgel GF_{254} with 60 ml distilled water. The slurry was spread in a layer 0.25 mm thick on 20 x 20 cm glass plates. The plates were dried in the open air and then activated at 105°C for one hour. Silver nitrate impregnated plates were prepared by mixing 30 g Kieselgel G with 60 ml of a 10% w/v solution of silver nitrate in distilled water. The slurry was spread in a layer as before.

(b) Solvent systems

The solvents used for elution of columns and the development of TLC plates are detailed in the appropriate sections.

(c) Detection of components on TLC plates

Short wave ultraviolet light (254 nm) and spraying with 20% v/v sulphuric acid in distilled water followed by heating at 105°C for 10 minutes were used for the detection of components.

B INFRARED SPECTRA

Obtained in solid films, Nujol mulls, chloroform solutions or KBr discs using a Unicam SP 200 spectrometer.

C ULTRAVIOLET SPECTRA

Taken in spectroscopic ethanol at a pathlength of 1 cm using a Perkin-Elmer Lambda 3 UV/VIS spectrophotometer.

D MELTING POINT

Taken with an Electrothermal melting point apparatus and are uncorrected.

E HYDROGEN-1 NUCLEAR MAGNETIC RESONANCE SPECTRA

Recorded at 400 MHz using a Bruker WH 400 spectrometer or at 200 MHz on a Nicolet NT 200 spectrometer in deuterochloroform using tetramethylsilane (TMS) as internal standard.

F CARBON-13 NUCELAR MAGNETIC RESONANCE SPECTRA

Recorded at 100.6 MHz using a Bruker WH 400 spectrometer in deuterochloroform using tetramethylsilane as internal standard.

Both ^{1}H and ^{13}C resonances are given in δ (TMS δ 0.0) and the following abbreviations have been used: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet and J, coupling constant.

G MASS SPECTRA

Recorded on an AEI MS 902 high resolution mass spectrometer or a VG Micromass 16S spectrometer at 18 or 70 eV with direct inlet temperatures of between 180°C and 240°C.

2 EXTRACTION OF CHRYSANTHEMUM PARTHENIUM WITH LIGHT PETROLEUM (b.r. 40-60°C)

5.8 kg of the leaves of Chrysanthemum parthenium were collected in January 1980 from the Chelsea Physic Garden, Royal Hospital Road, London SW3 and freeze-dried using a Chemical Laboratory Instruments SB4 freeze-drier. A voucher specimen of the dried aerial parts of the plant was placed in the museum at Chelsea College. The freeze-dried leaves lost 82.8% on drying to give a final weight of 1 kg. These were powdered and exhaustively extracted in a Soxhlet apparatus with light petroleum b.r. 40-60°C for 7 days. The extract was evaporated to dryness using a rotary evaporator under reduced pressure to give a yellow oily residue weighing 44 g.

3 SPASMOLYTIC ACTIVITY OF THE LIGHT PETROLEUM EXTRACT

50 mg of the petroleum extract was tested for spasmolytic activity using acetylcholine (Ach), 5-hydroxytryptamine (5-HT) and histamine as agonists as described below.

Guinea pigs weighing 200-300 g were killed by a blow on the head. The proximal ileum was excised and 2-3 cm lengths were cleaned and suspended in Krebs solution (NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.5 mMole/litre) at 37°C through which a mixture of 95% O_2 and 5% CO_2 was constantly bubbled. The lengths of ileum were set up to record longitudinal contractions isometrically. Log (dose) vs response curves were recorded to acetylcholine, 5-hydroxytryptamine and histamine from which ED_{50} doses were taken and given repeatedly until constant responses were achieved. The antagonist i.e. the petroleum extract, was then dissolved in the minimum of dimethylsulphoxide and made up to a

concentration of 10^{-4} g/ml with Krebs solution. This was added to the bath containing the ileum and left for 30 minutes. After thorough washing with Krebs solution to remove the residual antagonist the responses of the tissue to the agonists were then recorded and the percentage change calculated.

A control experiment was performed in exactly the same way except the antagonist was omitted. (This was essential since leaving an undosed tissue for 30 minutes often increases its sensitivity to exogenous agonist). The petroleum extract showed 100% inhibition to the three agonists.

4 CRUDE SEPARATION OF THE LIGHT PETROLEUM EXTRACT

1.25 g of the petroleum extract was taken and chromatographed on a silica gel column (50 g) to investigate the components present and the degree of separation in view of a larger scale examination. This pilot study proved satisfactory and so 40 g of the petrol extract was placed on a column containing 1.2 kg silica gel mixed to a slurry with light petroleum b.r. 40-60°. 200 ml fractions were collected. The fractions were examined by TLC and combined as appropriate.

Solvent elution of the column and weights of the combined fractions are shown in Tables 9 and 10.

5 SPASMOLYTIC ACTIVITY OF THE COMBINED FRACTIONS FROM THE LIGHT PETROLEUM EXTRACT

The combined fractions from the petroleum extract were tested for spasmolytic activity as described before (Part III, 3). The results

Table 9

Solvent elution of the column chromatography of the light petroleum extract

Fraction numbers	Solvent
1 - 24	light petroleum b.r. 40^{o}-60^{o}
25 - 36	1.0% v/v ethyl acetate in light petroleum
37 - 50	2.5% v/v ethyl acetate in light petroleum
51 - 65	5.0% v/v ethyl acetate in light petroleum
66 - 95	7.5% v/v ethyl acetate in light petroleum
96 - 112	10.0% v/v ethyl acetate in light petroleum
113 - 118	25.0% v/v ethyl acetate in light petroleum
119 - 166	50.0% v/v ethyl acetate in light petroleum
167 - 174	75.0% v/v ethyl acetate in light petroleum
175 - 180	ethyl acetate
181 - 185	1.0% v/v chloroform in ethyl acetate
186 - 188	5.0% v/v chloroform in ethyl acetate
189 - 192	10.0% v/v chloroform in ethyl acetate
193 - 195	25.0% v/v chloroform in ethyl acetate
196 - 201	50.0% v/v chloroform in ethyl acetate
202 - 217	chloroform
218 - 237	10.0% v/v methanol in chloroform
238	50.0% v/v methanol in chloroform

Table 10

Weights of combined fractions from the column chromatography of the light petroleum extract

Combined fractions (fraction numbers)	Weight (grams)	% of total (corrected to 1 decimal place)
1 - 8	2.995	7.5
9 - 26	2.692	6.7
27 - 50	0.380	1.0
51 - 61	5.850	13.7
62 - 70	1.170	2.9
71 - 74	0.230	0.6
75 - 80 (C)*	0.700	1.8
81 - 84	1.800	4.5
85 - 86	0.764	1.9
87 - 92	2.380	6.0
93 - 101	1.520	3.8
102 - 111	1.878	4.7
112 - 118 (F)*	1.409	3.5
119 - 123 (E)*	1.743	4.4
124 - 143 (B)*	7.625	19.1
144 - 176 (A)*	2.059	5.2
177 - 200 (D)*	0.796	2.0
201 - 228	1.646	4.1
229 - 238	2.046	5.1
	39.633	98.5

* Letters refer to combined fractions indicated in Figure 22.

are shown in Table 11 and Figure 22.

6 EXTRACTION OF CHRYSANTHEMUM PARTHENIUM WITH CHLOROFORM

The plant material, 950 g, previously extracted with light petroleum b.r. 40–60°C was exhaustively extracted with chloroform in a Soxhlet apparatus for 9 days. The extract was evaporated to dryness under reduced pressure using a rotary evaporator under reduced pressure to give a green oily residue weighing 46 g.

7 SPASMOLYTIC ACTIVITY OF THE CHLOROFORM EXTRACT

50 mg of the chloroform extract was tested for spasmolytic activity as before (Part III, 3). 100% inhibition of the three agonists was obtained.

8 CRUDE SEPARATION OF THE CHLOROFORM EXTRACT

4.5 g of the chloroform extract was chromatographed on a silica gel column (120 g) to investigate the components present and the degree of separation in view of a larger scale examination. 100 ml fractions were taken and combined as appropriate with reference to TLC. Solvent elution of the column and weights of the combined fractions are shown in Tables 12 and 13.

9 SPASMOLYTIC ACTIVITY OF THE COMBINED FRACTIONS FROM THE CHLOROFORM EXTRACT

The combined fractions from the chloroform extract were tested for

Table 11

Spasmolytic activity of the combined fractions from the light petroleum extract, tested at 10^{-4} g/ml

Fraction numbers	% inhibition of		
	Ach	5-HT	Histamine
1 - 8	0	0	0
9 - 26	0	0	0
27 - 50	20	31	33
51 - 61	89	100	96
62 - 70	26	32	26
71 - 74	55	87	50
75 - 80 (C)*	91	100	100
81 - 84	0	0	0
85 - 86	12	27	26
87 - 92	5	42	39
93 - 101	60	86	79
102 - 111	58	62	66
112 - 118 (F)*	20	42	35
119 - 123 (E)*	84	97	97
124 - 143 (B)*	91	100	100
144 - 176 (A)*	100	100	100
177 - 200 (D)*	81	100	100
201 - 228	50	57	46

* Letters refer to combined fractions indicated in Figure 22.

Table 12

Solvent elution of the column chromatography of the chloroform extract

Fraction numbers	Solvent
1 - 12	50.0% v/v ethyl acetate in light petroleum
13 - 16	75.0% v/v ethyl acetate in light petroleum
17 - 27	ethyl acetate
28 - 32	10.0% v/v chloroform in ethyl acetate
33 - 36	20.0% v/v chloroform in ethyl acetate
37 - 40	50.0% v/v chloroform in ethyl acetate
41 - 45	chloroform
46 - 54	10.0% v/v methanol in chloroform
55 - 62	25.0% v/v methanol in chloroform
63 - 68	50.0% v/v methanol in chloroform

Table 13

Weights of combined fractions of the column chromatography of the chloroform extract

Combined fractions (fraction numbers)	Weight (grams)	% of total (corrected to 1 decimal place)
1 - 2	0.429	9.5
3 - 4	0.417	9.3
5 - 6	0.171	3.8
7 - 8	0.097	2.2
9 - 12	0.201	4.5
13 - 18	0.362	8.1
19 - 22	0.273	6.1
23 - 25	0.410	9.2
26 - 39	0.264	5.9
40 - 54	0.940	20.9
55 - 68	0.805	17.9
	4.369	97.4

spasmolytic activity as described before (Part III, 3). The results are shown in Table 14. No fractions showed 100% antagonism to all three agonists and so were not investigated further with respect to pharmacological activity. The extract was fractionated however to isolate new compounds in the hope that these would be of help in the structural elucidation of the active ones from the light petroleum extract.

10 SEPARATION OF THE CHLOROFORM EXTRACT ON A LARGER SCALE

39 g of the chloroform extract was chromatographed on a column of silica gel (1 kg). 200 ml fractions were taken and combined as appropriate with reference to TLC. Solvent elution of the columns and weight of the combined fractions are shown in Tables 15 and 16.

11 EXTRACTION OF CHRYSANTHEMUM PARTHENIUM WITH METHANOL

A portion of the plant material, 25 g, previously extracted with light petroleum and $CHCl_3$ was extracted with methanol in a Soxhlet for 8 days. After evaporation of the solvent a black residue (4.6 g) was obtained.

12 SPASMOLYTIC ACTIVITY OF THE METHANOL EXTRACT

50 mg of the methanol extract was tested for spasmolytic activity as before (Part III, 3). 0% inhibition of the three agonists was obtained.

Table 14

Spasmolytic activity of the combined fractions from the chloroform extract, tested at 10^{-4} g/ml

Fraction numbers	% inhibition of		
	Ach	5-HT	Histamine
1 - 2	9	0	26
3 - 4	46	50	100
5 - 6	91	94	93
7 - 8	60	65	78
9 - 12	52	54	63
13 - 18	22	33	80
19 - 22	0	0	0
23 - 25	0	0	0
26 - 39	43	32	16
40 - 54	48	47	35
55 - 68	23	37	14

Table 15

Solvent elution of the column chromatography of the chloroform extract

Fraction number	Solvent
1 - 16	light petroleum
17 - 21	2.5% v/v chloroform in light petroleum
22 - 28	5.0% v/v chloroform in light petroleum
29 - 46	10.0% v/v chloroform in light petroleum
47 - 73	15.0% v/v chloroform in light petroleum
74 - 180	20.0% v/v chloroform in light petroleum
181 - 210	25.5% v/v chloroform in light petroleum
211 - 248	30.0% v/v chloroform in light petroleum
249 - 273	40.0% v/v chloroform in light petroleum
274 - 304	50.0% v/v chloroform in light petroleum
305 - 335	60.0% v/v chloroform in light petroleum
336 - 350	75.0% v/v chloroform in light petroleum
351 - 370	chloroform
371 - 378	1.0% v/v methanol in chloroform
379 - 467	2.5% v/v methanol in chloroform
468 - 501	5.0% v/v methanol in chloroform
502 - 576	10.0% v/v methanol in chloroform
577 - 584	20.0% v/v methanol in chloroform
585 - 633	40.0% v/v methanol in chloroform
634 - 646	60.0% v/v methanol in chloroform
647	methanol

Table 16

Weights of combined fractions of the column chromatography of the chloroform extract

Combined fractions (fraction number)	Weight (grams)	% of total (corrected to 1 decimal place)
1 - 55	0.209	0.5
56 - 130	0.746	1.9
131 - 153	2.161	5.5
154 - 158	0.266	0.7
159 - 160	0.183	0.5
161 - 199	1.540	3.9
200 - 247	3.105	8.0
248 - 267	1.211	3.1
268 - 278	0.672	1.7
279 - 300	2.396	6.1
301 - 307	0.410	1.1
308 - 328	1.745	4.5
329 - 339	0.469	1.2
340 - 358	1.646	4.2
359 - 382	1.475	3.8
383 - 384	1.482	3.8
385 - 387	1.172	3.0
388 - 393	1.970	5.1
394 - 397	1.259	3.2
398 - 401	1.668	4.3
402 - 409	1.473	3.8
410 - 423	1.826	4.7
424 - 440	1.474	3.8
441 - 469	1.561	4.0
470 - 489	2.344	6.0
490 - 514	2.097	5.4
515 - 530	1.377	3.5
531 - 582	1.073	2.8
583 - 658	0.605	1.5
	39.615	101.6

13 EXTRACTION OF CHRYSANTHEMUM PARTHENIUM WITH WATER

A portion of the plant material, 25 g, previously extracted with light petroleum, chloroform and methanol was exhaustively extracted with water in a Soxhlet for 7 days. After evaporation of the solvent a black residue (3.9 g) was obtained.

14 SPASMOLYTIC ACTIVITY OF THE WATER EXTRACT

50 mg of the water extract was tested for spasmolytic activity as before (Part III, 3). 0% inhibition of the three agonists was obtained.

15 STUDIES ON FRACTIONS 144-176 (A) FROM THE LIGHT PETROLEUM EXTRACT

Fractions 144-176 from the petroleum extract showed 100% inhibition of the three agonists Ach, 5-HT and histamine at 10^{-4} g/ml.

A FURTHER SEPARATION OF FRACTIONS 144-176 (A)

These fractions (1.959 g) were chromatographed on a silica gel column (40 g). 100 ml fractions were taken and combined as appropriate with reference to TLC. Solvent elution of the column and weight of the combined fractions are shown in Tables 17 and 18.

B SPASMOLYTIC ACTIVITY OF FRACTIONS 144-176 (A)

Those fractions weighing more than 50 mg were tested for spasmolytic activity as described before (Part III, 3). The

Table 17

Solvent elution of column chromatography of fractions 144-176 (A) from the light petroleum extract.

Fraction number	Solvent
A 1 - 7	hexane
A 8 - 25	10% v/v ethyl acetate in hexane
A 26 - 50	20% v/v ethyl acetate in hexane
A 51 - 65	40% v/v ethyl acetate in hexane
A 66 - 78	60% v/v ethyl acetate in hexane
A 79 - 85	ethyl acetate
A 86	chloroform

Table 18

Weights of combined fractions of column chromatography of fractions 144-176 (A) from the light petroleum extract

Fraction number	Weight (grams)	% total (corrected to 1 decimal place)
A 1 - 7	0.022	1.1
A 8 - 9	0.014	0.7
A 10 - 18	0.017	0.9
A 19 - 24	0.048	2.5
A 25 - 29	0.096	4.9
A 30 - 34	0.211	10.8
A 35 - 36	0.146	7.5
A 37 - 38	0.102	5.2
A 39 - 46	0.262	13.4
A 47 - 52	0.177	9.0
A 53 - 58	0.218	11.1
A 59 - 65	0.267	13.6
A 66 - 67	0.104	5.3
A 68 - 72	0.125	6.4
A 73 - 85	0.156	8.0
	1.965	100.2

results are shown in Table 19.

C FURTHER INVESTIGATION OF THE FRACTIONS SHOWING 100% INHIBITION OF THE THREE AGONISTS

(a) Fractions A 59-65

Since fractions A 59-65 weighed the most of the 100% active fractions this was investigated first.

It was decided to separate fractions A 59-65 by preparative TLC. 204 mg was placed on four 20 x 20 cm TLC plates (0.25 mm thickness) and developed in chloroform:methanol (98:2).

Eight bands were located under ultraviolet light (254 nm). The components in each band were extracted with chloroform in the usual way.

The major components of fractions A 59-65, i.e. band 6, crystallised from chloroform and methanol as fine needles (26 mg) of DJ140a, 8'α-[4'α,5'β-epoxy-1'β-(1β,10β-epoxy-4α-hydroxygermacr-11(13)-en-12, 6α-olactoyl-5β-yloxy)-germacra-10'(14'), 11'(13')-dien-12', 6'α-olactoyl]-8''α-(3a-hydroxy-3a, 4a-dimethyl-2a-methylenepentanoyl)-4''α,5''β-epoxy-1''β, 6''α-dihydroxygermacra-10''(14''), 11''(13'')-dien-12''oate (chrysanthemonin, 104), m.p. 211° (d); UV: λ_{max}^{EtOH}(log ε) 210 (4.01) nm; IR: $\nu_{max}^{Solid\ film}$ 3470 (hydroxyl), 1760 (>C=O, γ-lactone, 1715 (>C=O, ester), 1665 (>C=C<, conjugated), 1640 (>C=C<), 1265 (-C-O-C-, epoxide) cm^{-1}; ^1H NMR: δ (400 MHz, $CDCl_3$), 1.07 (3H, d, J = 7.0 Hz, H-5as or 6as), 1.21 (3H, d, J = 7.0 Hz, H-6as or 5as), 1.32 (3H, s, H-15s), 1.33 (3H, s, H-7as), 1.43 (3H, s, H-15's), 1.44 (3H, s, H-15''s), 1.59

Table 19

Spasmolytic activity of fractions 144-176 (A) from the light petroleum extract, tested at 10^{-4} g/ml.

Fraction number	% inhibition of		
	Ach	5-HT	Histamine
A 25 - 29	77	100	87
A 30 - 34	60	48	55
A 35 - 36	94	100	100
A 37 - 38	97	98	97
A 39 - 46	83	93	92
A 47 - 52	100	100	100
A 53 - 58	100	100	100
A 59 - 65	100	100	100
A 66 - 67	90	100	100
A 68 - 72	87	90	92
A 73 - 85	75	80	70

(3H, s, H-14s), 1.97 (1H, dd, J = 8.0, 2.0 Hz, H-9'), 2.03 (1H, m, H-9''), 2.18 (1H, d, J = 10.0 Hz, H-5), 2.38 (1H, d, J = 9.5 Hz, H-5''), 2.42 (1H, d, J = 11.5 Hz, H-5'), 2.58 (1H, m, H-3a), 2.82 (1H, dd, J = 9.0, 9.0 Hz, H-7'), 2.89 (1H, dd, J = 9.5, 9.5 Hz, H-7''), 2.99 (1H, m, H-1''), 3.08 (1H, m, H-7), 3.36 (1H, s, H-1), 3.99 (1H, m, H-1'), 4.01 (1H, dd, J = 11.5, 9.0 Hz, H-6'), 4.09 (1H, dd, J = 9.5, 9.5 Hz, H-6''), 4.13 (1H, dd, J = 10.0, 10.0 Hz, H-6), 4.78 (1H, d, J = 2.0 Hz, H-14'), 4.81 (1H, d, J = 2.0 Hz, H-14''), 5.09 (1H, d, J = 2.0 Hz, H-14'), 5.12 (1H, d, J = 2.0 Hz, H-14''), 5.19 (1H, m, H-8'), 5.33 (1H, d, J = 3.5 Hz, H-13a), 5.36 (1H, m, H-8''), 5.39 (1H, d, J = 3.5 Hz, H-13''a), 5.94 (2H, m, H-8a), 6.05 (1H, dd, J = 5.5, 1.0 Hz, H-13'a), 6.07 (1H, d, J = 3.5 Hz, H-13b), 6.09 (1H, dd, J = 5.5, 1.0 Hz, H-13'b), 6.13 (1H, d, J = 3.5 Hz, H-13''b); MS: m/z (%, rel. int.), 381 (1.7), 367 (2.0), 273 (2.1), 261 (1.7), 259 (1.9), 247 (2.4), 246 (8.1), 245 (4.7), 243 (11.0), 229 (13.0), 228 (39.0), 226 (14.2), 213 (12.2), 202 (6.3), 201 (10.7), 200 (10.2), 199 (6.0), 198 (5.2), 197 (5.7), 185 (7.0), 183 (9.1), 173 (6.4), 169 (7.1), 167 (7.9), 157 (8.6), 156 (8.2), 97 (34.5), 95 (15.2), 94 (100.0), 83 (33.1), 71 (19.8), 43 (11.3).

(i) Hydrolysis of DJ140a

52 mg of DJ140a (combined with mother liquors) was dissolved in dioxan (5 ml) and a stoichiometric amount of K_2CO_3 (i.e. 245 microlitres of a solution containing 500 mg in 5 ml) was added. The reaction was monitored by TLC. After seven days with a gradual increase in temperature and addition of more base only starting material could be detected.

2 ml of a 2% solution of KOH in H_2O was then added and the solution refluxed for 30 minutes. After normal work up the mixture was separated by preparative TLC using $CHCl_3$: MeOH (49:2) as developing solvent. More than twenty bands were located by ultraviolet light (254 nm) and spraying the edge of the plate with 20% v/v sulphuric acid and heating. The major component, located in band 2, was extracted with $CHCl_3$. DJ140a (i.e. the starting material) was located in band 3 and this was extracted and hydrolysed again using 2% w/v KOH solution and worked up as before. The organic solvent-soluble portion of this and the extract from band 2 of the original hydrolysis were identical on TLC. They were combined and purified by TLC using $CHCl_3$: MeOH (98:2) as developing solvent. The plate was developed three times. The major component was located in band 2 by spraying the edge of the plate with sulphuric acid as before and heating.

This component was further purified by TLC using $CHCL_3$: MeOH (98:2) as developing solvent. After extraction into $CHCl_3$ the material crystallised from $CHCl_3$ and hexane as colourless plates (5 mg) of DJ 140aH2, 4'α,5'β-epoxy-8'α-hydroxy-1'β-(1β,10β-epoxy-4α-hydroxygermacr-11(13)-en-12,6α-olactoyl-5β-yloxy)-germacra-10'(14'), 11'(13')-dien-12',6'α-olactone (102), m.p. 159° (d); UV: λ_{max}^{EtOH} (log ε), 209 (3.99) nm; IR: $\nu_{max}^{Solid\ film}$, 3500 (OH), 1760 (>C=O, γ-lactone), 1665 (>C=C<, conjugated), 1640 (>C=C<), 1260 (-C-O-C-, epoxide) cm^{-1}; ^1H NMR: δ (400 MHz, $CDCl_3$), 1.32 (3H, s, H-15s), 1.40 (3H, s, H-15's), 1.48 (1H, m, H-8), 1.55 (3H, s, H-14s), 1.77 (1H, m, H-9'), 2.06 (1H, m, H-9'), 2.23 (1H, m, H-8), 2.30 (1H, d, J = 14.5 Hz), 2.44 (1H, m, H-7'), 2.46 (1H, d, J = 11.5 Hz, H-5'), 2.59 (1H, dd, J = 14.5, 5.0 Hz), 2.68

(1H, d, J = 9.5 Hz, H-5), 3.01 (1H, m, H-8'), 3.20 (1H, m, H-7), 3.35 (1H, s, H-1), 3.92 (1H, dd, J = 11.5, 8.0 Hz, H-6'), 4.03 (1H, m, H-1'), 4.13 (1H, dd, J = 9.5, 9.5 Hz, H-6), 4.91 (1H, d, J = 1.5 Hz, H-14'), 5.15 (1H, d, J = 1.5 Hz, H-14'), 5.35 (1H, d, J = 3.5 Hz, H-13a), 5.89 (1H, dd, J = 5.5, 0.7 Hz, H-13'a), 6.07 (1H, dd, J = 5.5, 0.7 Hz, H-13'b), 6.09 (1H, d, J = 3.5 Hz, H-13b); ^{13}C NMR: δ (100.6 MHz, $CDCl_3$), 15.10, 18.69 and 29.10 (C-14, C-15 and C-15'), 23.40, 33.35, 34.64, 38.09, 38.77, 65.04, and 66.08 (C-2, C-2', C-3, C-3', C-8, C-9 and C-9'), 43.24 and 44.31 (C-7 and C-7'), 50.72 (C-8'), 55.30, 61.39 and 73.27 (C-4, C-10 and C-4'), 50.86, 62.90, 65.66 and 68.88 (C-1, C-1', C-5 and C-5'), 75.35 and 79.95 (C-6 and C-6'), 118.00 and 118.64 (C-13 and C-13'), 135.69 (C-14'), 137.92 (C-10'), 140.98 (C-11), 170.60 and 178.64 (C-12 and C-12'). C-11' not seen; MS: m/z (%, rel.int.), 430 (1.1), 356 (1.3), 342 (1.3), 281 (2.7), 281 (1.0), 280 (4.3), 279 (12.9), 266 (1.4), 265 (7.1), 261 (1.3), 247 (2.7), 228 (5.0), 207 (8.9), 167 (15.4), 150 (10.5), 149 (100), 113 (5.3), 99 (6.1), 98 (5.4), 97 (6.4), 94 (9.7), 87 (5.5), 85.1 (11.1), 85.0 (30.6), 83.1 (7.8), 83.0 (46.0), 71 (18.4), 70 (10.5), 69 (15.2), 60 (5.6), 57 (50.7), 56 (16.9), 55 (22.9); Accurate mass measurements: Found: 265.1449; $C_{15}H_{21}O_4$ requires 265.1440; Found: 279.1241; $C_{15}H_{19}O_5$ requires 279.1232.

(b) Fractions A 53-58 and A 66-67

These two sets of fractions were combined (0.236 g) and chromatographed on a silica gel column (10 g). 100 ml fractions were taken and combined as appropriate with reference to TLC. Solvent elution of the column and weights of the combined fractions are shown in Tables 20 and 21.

Table 20

Solvent elution of column chromatography of fractions A 53 - 58 and A 66-67 from the light petroleum extract

Fraction number	Solvent
Aa 1 - 19	2.5% v/v chloroform in hexane
Aa 20 - 22	5% v/v chloroform in hexane
Aa 23 - 30	10% v/v chloroform in hexane
Aa 31 - 63	20% v/v chloroform in hexane
Aa 64 - 98	30% v/v chloroform in hexane

Table 21

Weights of combined fractions of column chromatography of fractions A 53-58 and A 66-67 from the light petroleum extract

Fraction number	Weight	% of total
Aa 1 - 46	0.007	3.0
Aa 47 - 49	0.019	8.1
Aa 50 - 63	0.039	16.5
Aa 64 - 69	0.048	20.3
Aa 70 - 76	0.030	12.7
Aa 77 - 93	0.056	23.7
Aa 94 - 98	0.028	11.9
	0.227	96.2

Fractions Aa 70-76 contained only one major component which crystallised from chloroform and methanol to give 14 mg of DJ140a, identical with that obtained from fractions A 59-65, above.

Fractions Aa 50-63 (0.039 g) were further separated by preparative TLC using chloroform:methanol (98:2) as the developing solvent. Three bands were located under ultraviolet light (254 nm) and their constituents extracted with chloroform. The major components of fractions Aa 50-63, i.e. in band 2, weighed 18 mg and crystallised from chloroform and hexane as colourless needles (8 mg) of DJ177b, 9,10-epoxy-3, 5-dihydroxyguia-4(15), 11(13)-dien-12, 6α-olactone (chrysanthemolide, 93), m.p. 116-117° (d.); UV:λ_{max}^{EtOH} (log ε) 215 (4.10) nm; IR: $\nu_{max}^{solid\ film}$, 3540 (OH), 1760 (>C=O, γ-lactone), 1665 (>C=C<, conjugated), 1640 (>C=C<), 1250 (-C(-)-O-C(-)-, epoxide) cm^{-1}; ^1H NMR: δ (400 MHz, $CDCl_3$), 1.57 (3H, s, H-14s), 1.73 (2H, m, H-9s), 1.98 (2H, m, H-8s), 2.18 (2H, m, H-2s), 2.31 (1H, dd, J = 13.5, 11.0 Hz, H-7), 2.81 and 3.97 (1H each, br ss, C-3 and C-5 OHs), 4.41 (1H, m, H-3), 4.88 (1H, s, H-15a), 5.21 (1H, s, H-15b), 5.23 (1H, d, J = 11.0 Hz, H-6), 5.45 (1H, d, J = 3.5 Hz, H-13a), 6.18 (1H, d, J = 3.5 Hz, H-13b); MS: m/z (% rel. int.), 280 (2.0), 279 (2.6), 278 (0.2), 267 (3.4), 266 (5.5), 265 (16.8), 264 (40.8), 263 (17.9), 262 (6.2), 258 (4.9), 260 (8.0), 258 (4.9), 230 (22.0), 226 (10.5), 223 (7.2), 222 (18.2), 220 (10.7), 180 (18.6), 179 (11.9), 169 (10.9), 166 (13.2), 165 (18.0), 164 (14.7), 163 (14.6), 161 (10.4), 157 (20.4), 155 (14.1), 153 (10.7), 152 (19.2), 151 (21.9), 150 (24.7), 143 (24.0), 137 (32.9), 135 (21.1), 129 (20.1), 125 (25.8), 124 (30.1), 123 (37.4), 111 (43.4), 110 (45.4), 109 (46.6), 101

(54.0), 97 (68.1), 95 (74.3), 85 (58.9), 84 (65.7), 83 (100.0), 81 (64.0), 69 (68.7); Accurate mass measurement: Found 278.1150; $C_{15}H_{18}O_5$ requires 278.1154.

(i) Acetylation of DJ177b

4 mg of DJ177b was acetylated in the normal way using pyridine and acetic anhydride. After the usual work up spots for both starting material and a less polar product were present on TLC examination. The material was too little to be completely purified but the ^1H NMR spectrum of the crude product showed te following addditional signals which may be assigned to the actetate product: δ, $CDCl_3$: 2.16 (3H, s, CH_3CO_2-) and 4.60 (1H, m, H-3).

(c) Fractions A47-52

Fractions 47-52 (0.123 g) were chromatographed on a silica gel column (10 g). 100 ml fractions were taken and combined as appropriate with reference to TLC. Solvent elution of the column and weight of the combined fractions are shown in Tables 22 and 23.

Fractions Ab 34-56 were further separated by preparative TLC using chloroform:methanol (98:2) as the developing solvent. Four bands were located under ultraviolet light (254 nm) and their components extracted with chloroform. The major component, i.e. contained in band 3, weighed 20 mg and crystallised from chloroform and n-pentane to give DJ179c. This material was a mixture of sesquiterpene lactones which resisted all attempts at purification. It was thus not investigated further.

Table 22

Solvent elution of column chromatography of fractions A47-52 from the light petroleum extract

Fraction number	Solvent
Ab 1 - 8	10% v/v chloroform in hexane
Ab 9 - 20	20% v/v chloroform in hexane
Ab 21 - 56	25% v/v chloroform in hexane
Ab 57	chloroform

Table 23

Weights of the combined fractions of column chromatography of fractions A47-52 from the light petroleum extract

Fraction number	Weight (grams)	% total
Ab 1 - 19	0.015	12.2
Ab 20 - 23	0.004	3.3
Ab 24 - 33	0.039	31.7
Ab 34 - 56	0.053	43.0
Ab 57	0.007	5.7
	0.118	95.9

The components in band 1 (11 mg) were further purified by preparative TLC. The plate was run seven times with chloroform : methanol (98:2) as the developing solvent. Four bands were located under ultraviolet light (254 nm) and their components extracted with chloroform. The components contained in band 1 of the latter plate weighed 6 mg and crystallised from chloroform and n-pentane to give DJ179a1. This material was possibly also a mixture of sesquiterpene lactones despite showing only one spot on TLC investigation. Signals were seen in the ^{1}H NMR spectrum which could be assigned to reynosin,[50] chrysartemin B[50,51] and perhaps a dihydro-derivative of reynosin. The paucity of material precluded further study at this stage but the further possibility that of the material's being a trimeric compound rather like DJ140a cannot be discounted.

16 STUDIES ON FRACTIONS 124-143 (B) FROM THE LIGHT PETROLEUM EXTRACT

Fractions 124-143 from the petroleum extract showed 100% inhibition ofthe two agonists 5-HT and histamine, and 91% inhibition of Ach.

A FURTHER SEPARATION OF FRACTIONS 124-143 (B)

These fractions (7.525 g) were chromatographed on a silica gel column (150 g). 100 ml fractions were taken and combined as appropriate with reference to TLC. Solvent elution of the column and weight of the combined fractions are shown in Tables 24 and 25.

Table 24

Solvent elution of column chromatography of fractions 124-143 (B) from the light petroleum extract

Fraction number	Solvent
B 1 - 27	hexane
B 28 - 45	1% v/v chloroform in hexane
B 46 - 51	2% v/v chloroform in hexane
B 52 - 60	5% v/v chloroform in hexane
B 61 - 67	10% v/v chloroform in hexane
B 68 - 80	20% v/v chloroform in hexane
B 81 - 136	40% v/v chloroform in hexane
B 137 - 149	60% v/v chloroform in hexane
B 150 - 170	75% v/v chloroform in hexane
B 171 - 194	chloroform
B 195 - 203	90% v/v chloroform in methanol
B 204	50% v/v chloroform in methanol

Table 25

Weights of combined fractions of column chromatography of fractions 124-143 (B) from the light petroleum extract.

Fraction number	Weight (grams)	% of total
B 1 - 9	0.011	0.1
B 10 - 90	0.040	0.5
B 91 - 106	0.025	0.3
B 107 - 111	2.484	33.0
B 112 - 115	1.537	20.4
B 116 - 122	0.490	6.5
B 123 - 130	0.316	4.2
B 131 - 147	0.252	3.3
B 148 - 163	0.454	6.0
B 164 - 171	0.200	2.6
B 172 - 196	0.810	10.8
B 197 - 198	0.682	9.1
B 199	0.078	1.0
B 200	0.083	1.1
B 201 - 204	0.048	0.6
	7.510	99.6

B SPASMOLYTIC ACTIVITY OF FRACTIONS 124-143 (B)

Those fractions weighing more than 50 mg were tested for spasmolytic activity as described before (Part III, 3). The results are shown in Table 26.

C FURTHER INVESTIGATION OF THE FRACTIONS SHOWING 100% INHIBITION OF THE THREE AGONISTS

Fractions B 107-111 showed 100% inhibition of all three agonists. The fractions (2.434 g) were chromatographed on a silica gel column (120 g). 100 ml fractions were taken and combined as appropriate with reference to TLC. Solvent elution of the column and weight of the combined fractions are shown in Tables 27 and 28.

The major component of fractions B 107 - 111, i.e. contained in subfractions Ba 34 - 55, crystallised from chloroform and hexane to give parthenolide (478 mg) as colourless plates, m.p. 113-114°; UV: λ_{max}^{EtOH} (log ε) 213 (4.20) nm; IR: ν_{max}^{KBr} 1760 (>C=O, γ-lactone), 1660 (>C=C<, conjugated), 1640 (>C=C<), 1250 (-$\overset{|}{\underset{|}{C}}$-O-$\overset{|}{\underset{|}{C}}$-) cm^{-1}; ^1H NMR: δ (400 MHz, $CDCl_3$), 1.32 (3H, s, H-15s), 1.73 (3H, s, H-14s), 2.81 (1H, d, J = 8.5Hz, H-5), 2.81 (1H, m, H-7), 3.87 (1H, dd, J = 8.5, 8.5 Hz, H-6), 5.23 (1H, dd, J = 12.5, 3.0 Hz, H-1), 5.64 (1H, d, J = 3.8 Hz, H-13a), 6.35 (1H, d, J = 3.8 Hz, H-13b); ^{13}C NMR: δ (100.6 MHz, $CDCl_3$), 16.97, 17.24 (C-14 and C-15), 24.11, 30.64 (C-8 and C-3), 36.35, 41.19 (C-2 and C-9), 47.65 (C-7), 61.45 (C-5), 66.36 (C-4), 82.38 (C-6), 121.08 (C-1), 125.24 (C-13), 134.51 (C-10), 139.21 (C-11), C-12 not detected; MS: m/z (%, rel. int.), 248 (1.5), 233 (1.1), 230 (8.1), 190 (9.3), 107 (8.4), 105 (12.2), 95 (8.9), 91 (13.6), 81 (12.4), 79 (11.7), 77 (9.2), 67 (9.6), 58 (24.8), 55 (12.0),

Table 26

Spasmolytic activity of fractions 124-143 (B) from the light petroleum extract, tested at 10^{-4} g/ml

Fraction number	% inhibition of		
	Ach	5-HT	Histamine
B 107 - 111	100	100	100
B 112 - 115	100	100	100
B 116 - 122	95	100	100
B 123 - 130	82	100	98
B 131 - 147	78	100	100
B 148 - 163	51	88	97
B 164 - 171	22	29	90
B 172 - 196	3	44	100
B 197 - 198	16	58	33
B 199	31	72	65
B 200	27	42	20

Table 27

Solvent elution of the column chromatography of fractions B 107 - 111 from the light petroleum extract

Fraction number	Solvent
Ba 1 - 5	10% chloroform in hexane
Ba 6 - 11	20% chloroform in hexane
Ba 12 -70	30% chloroform in hexane
Ba 71 - 73	chloroform

Table 28

Weights of combined fractions of column chromatography of fractions B 107 -111 from the light petroleum extract

Fraction number	Weight (grams)	% of total
Ba 1 - 20	0.112	4.6
Ba 21 -32	0.465	19.1
Ba 33	0.057	2.3
Ba 34 - 55	1.421	58.4
Ba 56 - 58	0.128	5.3
Ba 59 - 68	0.130	5.3
Ba 69 - 70	0.094	3.9
Ba 71 - 73	0.030	1.2
	2.437	100.1

53 (17.3), 43 (100.0). Lit.[40] m.p. 107-111°. Lit.[42] m.p. 115° This material was identical in all respects with an authentic sample supplied by F.Sorm.

17 STUDIES ON FRACTIONS 75-80 (C) FROM THE LIGHT PETROLEUM EXTRACT

Fractions 75-80 (C) from the petroleum extract showed 100 % inhibition of the two agonists, 5-HT and histamine, and 91% inhibition of Ach.

A FURTHER SEPARATION OF FRACTIONS 75-80 (C)

0.65 g were chromatographed on a column of silica gel (60 g). 100 ml fractions were collected and combined as appropriate with reference to TLC. The weights of the combined fractions and solvent elution of the column are given in Tables 29 and 30.

18 STUDIES ON FRACTIONS 177-200 (D) FROM THE PETROLEUM EXTRACT

Fractions 177-200 from the petroleum extract showed 100% inhibition of the two agonists 5-HT and histamine, and 81% inhibition of Ach.

A FURTHER SEPARATION OF FRACTIONS 177-200 (D)

These fractions (0.729 g) were chromatographed on a silica gel column (30 g). 100 ml fractions were taken and combined as appropriate with reference to TLC. Solvent elution of the column and weights of the combined fractions are shown in Tables 31 and 32.

B SPASMOLYTIC ACTIVITY OF FRACTIONS 177-200 (D)

Those fractions weighing more than 50 mg were tested for

Table 29

Solvent elution of column chromatography of fractions 75 - 80 (C) from the light petroleum extract

Fraction number	Solvent
C 1 - 3	5% chloroform in hexane
C 4 - 12	10% chloroform in hexane
C 13 - 29	20% chloroform in hexane
C 30 - 33	40% chloroform in hexane
C 34	chloroform

Table 30

Weights of combined fractions of column chromatography of fractions 75 - 80 (C) from the light petroleum extract

Fraction number	Weight (grams)	% total
C 1 - 15	0.020	3.1
C 16 - 19	0.036	5.5
C 20 - 24	0.446	68.6
C 25 - 26	0.044	6.8
C 27 - 39	0.103	15.8
	0.649	99.8

All these subfractions were devoid of spasmolytic activity at a concentration of 10^{-4} g/ml and so were not investigated further.

Table 31

Solvent elution of column chromatography of fractions 177-200 (D) from the light petroleum extract

Fraction number	Solvent
D 1 - 12	25% v/v ethyl acetate in hexane
D 13 - 17	40% v/v ethyl acetate in hexane
D 18 - 24	60% v/v ethyl acetate in hexane
D 25 - 31	80% v/v ethyl acetate in hexane
D 32 - 36	ethyl acetate
D 37 - 40	10% v/v chloroform in ethyl acetate
D 41 - 45	30% v/v chloroform in ethyl acetate

Table 32

Weights of combined fractions of column chromatography of fractions 177-200 (D) from the light petroleum extract.

Fraction number	Weight (grams)	% of total
D 1 - 3	0.037	5.1
D 4 - 8	0.090	12.3
D 9 - 10	0.049	6.7
D 11 - 15	0.088	12.1
D 16 - 20	0.240	32.9
D 21 - 26	0.160	21.9
D 27 - 45	0.070	9.6
	Total = 0.734	100.6

spasmolytic activity as described before (Part III, 3). The results are shown in Table 33.

C FURTHER INVESTIGATION OF THE FRACTIONS SHOWING 100% INHIBITION OF THE AGONISTS

(a) Fractions D 16-20

Fractions D 16-20 (0.175 g) were further separated by preparative TLC using chloroform:methanol (95:5) as developing solvent. The plates were run three times. Three bands were located under ultraviolet light (254 nm) and their components extracted with chloroform. The major component, i.e. in band 1, weighed 59 mg and crystallised from chloroform and methanol as colourless plates (26 mg) of DJ 154a, m.p. 248°; UV: λ_{max}^{EtOH} (log ε) 208 (3.98) nm; IR: ν_{max}^{KBr} 3420 (OH), 1760 (>C=O, γ-lactone), 1675 (>C=C<, conjugated), 1260 (-C-O-C-, epoxide) cm^{-1}; ^1H NMR: δ (400 MHz, $CDCl_3$), 1.15 (3H, s, H-15s), 1.58 (3H, s, H-14s), 2.87 (1H, d, J = 11.0 Hz, H-5), 3.30 (1H, m, H-7), 3.32 (1H, d, J = 1.3 Hz, H-3), 3.57 (1H, d, J = 1.3 Hz, H-2), 4.09 (1H, dd, J = 11.0, 10.0 Hz, H-6), 5.54 (1H, d, J = 3.5 Hz, H-13a), 6.19 (1H, d, J = 3.5 Hz, H-13b); MS: m/z (%, rel.int.) 279 (0.7), 278 (2.1), 263 (2.3), 262 (4.7), 260 (3.1), 249 (3.1), 231 (6.3), 217 (8.6), 203 (9.4), 189 (12.5), 175 (15.6), 165 (26.6), 151 (40.6), 149 (23.4), 147 (28.9), 123 (26.6), 121 (33.6), 112 (88.3), 109 (42.2), 97 (35.9), 95 (93.8), 91 (41.4), 85 (47.7), 83 (44.5), 81 (36.7), 79 (38.3), 77 (35.9), 71 (78.1), 69 (52.3), 67 (49.2), 57 (93.8), 55 (71.1), 53 (75.8), 43 (100.0). Lit.[50] m.p. 250° This material was identical in all respects with literature data on chrysartemin A.[50,51]

Table 33

Spasmolytic activity of fractions 177-200 (D) from the light petroleum extract, tested at 10^{-4} g/ml

Fraction number	% inhibition of		
	Ach	5-HT	Histamine
D 1 - 4	64	96	91
D 4 - 8	81	92	93
D 11 - 15	97	100	100
D 16 - 20	100	100	100
D 21 - 26	100	100	100
D 27 - 45	47	52	67

(b) Fractions D 21-26

Fractions D21-26 (0.110 g) were further separated by preparative TLC using chloroform:methanol (95:5) as developing solvent. The plates were run three times. Three bands were located under ultraviolet light (254 nm) and by spraying the edge of the plate with H_2SO_4 and heating. Their components were extracted with chloroform.

The major component of fractions D 21-26, i.e. in band 2, crystallised from chloroform and hexane to give colourless needles (16 mg) of DJ156a, 4α,10β-dihydroxy-1β,5α-gui-11(13)-en-12,6α-olactone (partholide, 82),m.p. 154°; UV: λ_{max}^{EtOH} (log ε) 208 (3.95) nm; IR: $\nu_{max}^{Solid\ film}$, 3490 (OH), 1750 (>C=O, γ-lactone), 1660 (>C=C<, conjugated), 1640 (>C=C<); ^{1}H NMR: δ (400 MHz, CDC_3), 1.25 (3H, s, H-14s), 1.35 (3H, s, H-15s), 1.47 (1H, m, H-8), 1.62 (2H, m, H-2s), 1.67 - 2.04 (6H, br m, H-3s, H-9s, 2 x OHs), 2.16 (1H, m, H-8), 2.39 (1H, dd, J = 12.3, 12.3 Hz, H-5), 2.63 (1H, m, H-1), 2.70 (1H, m, H-7), 4.24 (1H, dd, J = 12.3, 9.5 Hz, H-6), 5.54 (1H, d, J = 3.5 Hz, H-13a), 6.25 (1H, d, J = 3.5 Hz, H-13b); ^{13}C NMR: δ (100.6 MHz, $CDCl_3$), 23.51, 24.25 (C-14 and C-15), 25.01, 25.33 (C-2 and C-8), 39.34, 43.77 (C-3 and C-9), 47.22 (C-7), 49.78 (C-1), 55.35 (C-5), 74.72 (C-4), 79.88 (C-10), 82.74 (C-6), 120.37 (C-13), 138.45 (C-11), 169.37 (C-12); MS: m/z (% rel.int.), 266 (0.4), 265 (0.9), 264 (1.8), 263 (1.8), 262 (3.6), 261 (2.7), 260 (4.5), 249 (3.0), 248 (6.0), 247 (7.2), 246 (18.0), 245 (6.3), 244 (13.5), 232 (4.5), 231 (15.3), 230 (10.0), 229 (9.0), 228 (19.8), 215 (12.6), 213 (18.9), 203 (22.5), 191 (22.5), 190 (27.0), 188 (24.3), 175 (24.3), 173 (22.5), 159 (24.3), 105 (45.0), 95 (44.6), 93 (44.1), 91 (59.5), 83 (85.6), 81

(51.4), 79 (49.5), 71 (42.3), 69 (47.7), 57 (59.5), 55 (91.9), 43 (100.0); Accurate mass measurement: Found: 264.1364; $C_{15}H_{20}O_4$ requires 264.1362.

19 STUDIES ON FRACTIONS 119-123 (E) FROM THE LIGHT PETROLEUM EXTRACT

Fractions 119-123 from the petroleum extract showed 97% inhibition of 5-HT and histamine and 84% inhibition of Ach.

A FURTHER SEPARATION OF FRACTIONS 119-123 (E)

These fractions (1.583 g) were chromatographed on a silica gel column (30 g). 100 ml fractions were taken and combined as appropriate with reference to TLC. Solvent elution of the column and weights of the combined fractions are shown in Tables 34 and 35.

B SPASMOLYTIC ACTIVITY OF FRACTIONS 119 - 123 (E)

Those fractions weighing more than 50 mg were tested for spasmolytic activity as described before (Part III, 3). The results are shown in Table 36.

C FURTHER INVESTIGATION OF THE FRACTIONS SHOWING 100% INHIBITION OF THE AGONISTS

Fractions E 15 - 21 were combined and purified by TLC using chloroform : hexane (75:25) as developing solvent. The major component was extracted and identified as parthenolide by chromatographic comparison with an authentic specimen and by

Table 34

Solvent elution of column chromatography of fractions 119-123 (E) from the light petroleum extract

Fraction number	Solvent
E 1 - 2	hexane
E 3 - 5	10% v/v chloroform in hexane
E 6 - 31	20% v/v chloroform in hexane
E 32	chloroform

Table 35

Weights of combined fractions of column chromatography of fractions 119-123 (E) from the light petroleum extract

Fraction number	Weight (grams)	% of total
E 1 - 13	0.010	0.6
E 14	0.007	0.4
E 15	0.058	3.7
E 16 - 18	0.450	28.4
E 19 - 21	0.201	12.7
E 22 - 31	0.148	9.3
E 32 - 36	0.251	15.9
E 37 - 38	0.064	4.0
E 39	0.395	25.0
	1.584	100.0

Table 36

Spasmolytic activity of fractions 119-123 (E) from the light petroleum extract, tested at 10^{-4} g/ml

Fraction number	% inhibition of		
	Ach	5-HT	Histamine
E 15	67	97	91
E 16 - 18	57	96	89
E 19 - 21	85	100	100
E 22 - 31	28	100	92
E 32 - 36	85	100	98
E 37 - 38	56	100	100
E 39	78	100	91

spectroscopic means.

20 STUDIES ON FRACTIONS 112-118 (F) FROM THE LIGHT PETROLEUM EXTRACT

The major component of fractions 112-18 crystallised from chloroform to give DJ52A. The mass spectrum of this showed two compounds to be present with molecular ions at m/z 414 and 412. The mixture was acetylated with acetic anhydride in pyridine. The crude acetate mixture, obtained after the normal work up, was separated using argentation TLC with n-hexane:chloroform (90:10) as developing solvent. The plates were run five times. The two compounds were located by spraying the edge of the plate with 20% H_2SO_4 and extracted with chloroform to yield 196 mg and 157 mg of materials with melting points 126° and 141°. These were identical in all respects with authentic specimens of β-sitosterol and stigmasterol.

21 STUDIES ON FRACTIONS 51-61 FROM THE LIGHT PETROLEUM EXTRACT

Fractions 51-61 from the light petroleum extract showed 100% inhibition of 5-HT, 96% inhibition of histamine and 89% of Ach.

A FURTHER SEPARATION OF FRACTIONS 51-61

5.8g were chromatographed on a column of silica gel (100g). 100ml fractions were taken and combined as appropriate with reference to TLC. The solvent elution and weights of the combined fractions are shown in Tables 37 and 38.

Table 37

Solvent elution of column chromatography of fractions 51-61 from the light petroleum extract

Fraction number	Solvent
1-2	light petroleum
3-15	toluene
16-17	50% ethyl acetate in toluene
18	chloroform
19	methanol

Table 38

Weights of combined fractions of column chromatography of fractions 51-61 from the light petroleum extract

Fraction number	Weight (grams)	% total
1-4	3.091	53.3
5-6	1.291	22.3
7-15	1.291	22.3
16-19	0.064	1.1
	5.737	99.0

All these subfractions were devoid of spasmolytic activity at a concentration of 10^{-4} g/ml. From subfractions 7-15, DJ61a was isolated which proved to be mixture of long chain fatty acid esters.

22 PREPARATION OF CAPSULES FOR CLINICAL STUDIES

A CALCULATION OF DOSE

(a) Weights of fresh and freeze-dried leaves

52 fresh feverfew leaves approximately 1.5 inches long and 1.2 inches wide each containing 5 leaflets were weighed immediately after picking, freeze-dried and then re-weighed to constant weight. These weights are given in Table 39.

Weights of fresh leaf

Mean = 0.14908 g

Mean $\pm$ 2S = 0.20588 - 0.09228 g

The weights of all fresh leaves lay within two standard deviations of the mean.

Weights of dry leaf

Mean = 0.02566 g

Mean $\pm$ 2S = 0.036339 - 0.014981 g

The weights of all dry leaves lay within two standard deviations of the mean.

(b) The dose of dry leaf equivalent to that of fresh leaf

Patients using feverfew for migraine take between one and three fresh leaves daily in a bread and butter sandwich. One fresh leaf weighs approximately 150 mg which is equivalent to approximately 25 mg of freeze-dried leaf. Therefore, capules containing 25, 50 and 75 mg of freeze-dried feverfew, i.e. equivalent to 1, 2 or 3 fresh leaves, were formulated.

Table 39

Weights of feverfew leaves

Leaf number	Weight of fresh leaf (grams)	Weight of dry leaf (grams)	$\frac{\text{Dry weight}}{\text{Fresh weight}}$ %
1	0.17348	0.02821	16.26124
2	0.14703	0.02687	18.27518
3	0.14980	0.02425	16.18825
4	0.19009	0.03040	15.99242
5	0.17416	0.03131	17.97772
6	0.16650	0.03090	18.55855
7	0.12151	0.02255	18.55814
8	0.13768	0.02223	16.14613
9	0.12153	0.02344	19.28741
10	0.13678	0.02321	16.96885
11	0.14708	0.02310	15.70573
12	0.13092	0.02155	16.46043
13	0.11424	0.01905	16.67542
14	0.11441	0.02244	19.61367
15	0.14299	0.02625	18.35792
16	0.13021	0.02357	18.10152
17	0.11370	0.01743	15.32981
18	0.12300	0.02333	18.96747
19	0.13763	0.02860	20.78035
20	0.11712	0.02372	20.25273
21	0.14862	0.03149	21.18826
22	0.18080	0.03515	19.44137
23	0.12394	0.02576	20.78425
24	0.19835	0.03591	18.10436
25	0.11916	0.02133	17.90030
26	0.12021	0.02219	18.45936
27	0.19378	0.03341	17.24120

continued on next page

Table 39 (continued)

Leaf number	Weight of fresh leaf (grams)	Weight of dry leaf (grams)	Dry weight / Fresh weight %
28	0.11090	0.02027	18.27772
29	0.14977	0.02440	16.29164
30	0.12515	0.01875	14.98202
31	0.19099	0.03302	17.28886
32	0.13261	0.01838	13.86019
33	0.16754	0.02740	16.35430
34	0.20072	0.03397	16.92407
35	0.12833	0.01807	14.08088
36	0.19996	0.03227	16.13822
37	0.16540	0.02867	17.33373
38	0.14967	0.02257	15.07984
39	0.12571	0.01774	14.11184
40	0.20463	0.03629	17.56279
41	0.10423	0.01499	13.97828
42	0.16440	0.02614	15.90024
43	0.14735	0.02220	15.06616
44	0.14813	0.02344	15.82393
45	0.17356	0.02929	16.87600
46	0.12831	0.02247	17.51227
47	0.13057	0.02228	17.06364
48	0.17139	0.03173	18.51333
49	0.20492	0.03352	16.35760
50	0.15486	0.02604	17.26720
51	0.12881	0.02455	19.05907
52	0.16966	0.02823	16.63916
Total	7.75229	1.33433	
Mean	0.14908	0.02566	
S.D.	0.02840	0.00534	

B FORMULATION OF CAPSULES

Ingredients	25 mg capsules	50 mg capsules	75 mg capsules	placebo
Freeze dried feverfew	25	50	75	-
Lactose	145	130	105	q.s.
Chlorophyll	-	-	-	q.s.
Total	170 mg	180 mg	180 mg	

The slight end-weight differences were governed by the characteristics of the capsule filling machine. The capsules used were Eli Lilly, size 2, opaque, No. VX 2116 BAS. The average weight of the empty capsules was 60 mg.The ingredients of the capsules were granulated in the usual way with a 20 mesh sieve using 70% ethanol. Fines were removed by sieving with a 60 mesh sieve. Placebo capsules were prepared using lactose and chlorophyll to give granules identical in colour with those containing feverfew. The bottles containing the placebo capsules were sprinkled with a small amount of feverfew powder so that , on opening the bottles, both gave identical smell.

C BP TESTS ON THE CAPSULES [131a,b]

Capsule	Uniformity of weight[131a]	Disintegration test[131b]
25 mg	Average wt. 20 caps. = 171.8 mg. Greatest deviation from average = 9%	Disintegration time = 6.5 mins.
50 mg	Average wt. 20 caps. = 184.4 mg. Greatest deviation from average = 5%	Disintegration time = 5.3 mins.
75 mg	Average wt. 20 caps. = 183.9 mg. Greatest deviation from average = 7%	Disintegration time = 5.9 mins.
Placebo	Average wt. 20 caps. = 243.2 mg. Greatest deviation from average = 10%	Disintegration time = 6.0 mins.

All the capsules complied with both requirements.

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